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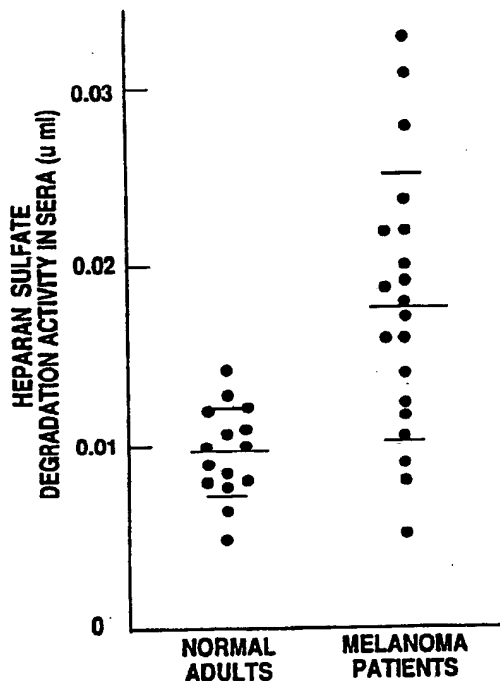
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(71) Applicant: BOARD OF REGENTS, THE UNIVERSITY OF TEXAS SYSTEM [US/US]; 201 West 7th Street, Austin, TX 78701 (US).		(81) Designated States: AT (European patent), AU, BE (European patent), CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FR (European patent), GB (European patent), GR (European patent), IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent).	
(72) Inventors: NICOLSON, Garth, L. ; 2611 Valley Manor, Kingwood, TX 77339 (US). NAKAJIMA, Motowo ; 5803 Dryad, Houston, TX 77035 (US). JIN, Li ; 1909 Brunson, Apt. 1, Houston, TX 77030 (US).		Published With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.	
(74) Agent: HODGINS, Daniel, S.; Arnold, White & Durkee, P.O. Box 4433, Houston, TX 77210 (US).			

(54) Title: IMMUNOCHEMICAL LOCALIZATION OF HEPARANASE IN MOUSE AND HUMAN MELANOMAS

(57) Abstract

Antibodies directed against N-terminal heparanase peptide are produced. These antibodies are used for detection of heparan sulfate endoglycosidase in human and murine tumors. Localization and mounts of these heparanase antigens may ultimately be used to design appropriate therapeutic courses.



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**IMMUNOCHEMICAL LOCALIZATION OF HEPARANASE
IN MOUSE AND HUMAN MELANOMAS**

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10 CROSS REFERENCES TO RELATED APPLICATION

 This application is a continuation-in-part application of copending U.S. patent application serial number 377,015 filed July 7, 1989, which is a divisional
15 of U.S. patent application serial number 012,860 filed February 20, 1987, which is a continuation-in-part of serial number 839,890, filed March 10, 1986.

 The immunochemical localization of the glycosamino
20 endoglycosidase, heparanase, in human and murine tumor types is an object of the present invention.

 The present invention also relates to an assay for endoglycosidase enzymic activity and a labeled substrate
25 for use in such an assay. The assay of the present invention is viewed as useful for the detection of cancerous malignancies.

 A class of biological substances called the pro-
30 teoglycans form the ground substance in the extracellular matrix of connective tissues. These proteoglycans are polyanionic substances of high molecular weight and contain many different types of heteropolysaccharide side chains covalently linked to a polypeptide backbone.
35 These proteoglycans may contain over 95% carbohydrates. The polysaccharide groups of the proteoglycans were formerly called mucopolysaccharides but now are preferably termed glycosaminoglycans since all contain derivatives of glucosamine or galactosamine.

A variety of enzymes may be involved in the normal metabolic degradation of proteoglycans. Initial proteoglycan degradation often involves proteolysis to separate or digest protein components. Such proteolysis results in the production of glycosaminoglycans. The glycosaminoglycans in turn are subject to glycosaminoglycan endoglycosidase enzymic action which produces smaller glycosaminoglycan fragments. The glycosaminoglycans or fragments thereof are subject to glycosaminoglycan exoglycosidase enzymic action which produces monosaccharides from the non-reducing ends of glycosaminoglycans.

An increasing interest in the endoglycosidases has arisen in recent years because of a possible relationship of these enzymes with tumor invasiveness and tumor metastatic activity. Nicolson (1982, Biochem. Biophys. Acta. V 695, pp 113-176) reviewed a variety of oligosaccharide-degrading enzymes (pp 141-142) reported to be of interest in malignant disease. Nicolson (1982, J. Histochem. & Cytochem. V 30, pp 214-220) described a proposed mechanism for tumor cell invasion of endothelial cell basal lamina and a related production of degradation products from proteins and glycosaminoglycans. Kramer et al. (1982, J. Biol. Chem. V 257, pp 2678-2686) reported a tumor-derived glycosidase capable of cleaving specifically glycosaminoglycans and releasing heparan sulfate-rich fragments.

Irimura et al. (1983, Analyt. Biochem. V 30, pp 461-468) describe high-speed gel-permeation chromatography of glycosaminoglycans. Heparan sulfate degrading activity of melanoma cells was measured by using this chromatographic procedure. Nakajima et al. (1983, Science, V 220, pp 611-613) described a relationship of

metastatic activity and heparan sulfate degrading activity in melanoma cell lines. The disappearance of higher molecular weight heparan sulfate was followed by polyacrylamide gel electrophoresis, staining and densitometry.

Vlodavsky et al. (1983, Cancer Res. V 43, pp 2704-2711) described the degradation by two T-lymphoma cell lines of ³⁵S labeled proteoglycans from confluent endo-
thelial cells. The highly metastatic line had much higher ³⁵S liberating activity than did the low metastatic line.

Irimura et al. (1983, Proc. Am. Soc. Cancer Res. V 24, p 37, abstract 144), using high performance liquid chromatography, describe heparan sulfate degradative enzyme activity of melanoma cells. Nakajima et al (1984, J. Biol. Chem. V 259, pp 2283-2290) describe characterizations of metastatic melanoma heparanase.
High speed gel permeation chromatography and chemical analyses were used in a description of functional substrates and products formed.

The background described herein involves an interest in convenient, accurate and reproducible endoglycosidase assays, particularly since endoglycosidases may play critical roles in the establishment of tumor metastases.

The ability of tumor cells to invade host tissues and metastasize to distant, often specific organ sites, is one of their most important properties. Metastasis formation occurs via a complex series of unique interactions between tumor cells and normal host tissues and cells. These processes involve several discrete and selective steps such as: invasion of surrounding tissues, penetration of lymphatics of blood vessels and

transport in lymph or blood, or dissemination into a serous cavity, arrest and invasion at distant sites, and survival and growth to form secondary lesions.

5 Basement membranes are continuous sheets of extra-cellular matrix composed of collagenous and non-collagenous proteins and proteoglycans that separate parenchymal cells from underlying interstitial connective
10 tissue. They have characteristic permeabilities and play a role in maintaining tissue architecture. Metastasizing tumor cells must penetrate epithelial and endothelial basement membranes during invasion and metastasis, and the penetration and destruction of basement membranes by
15 invasive tumor cells has been observed using electron microscopy. Since basement membranes are rigid structures formed from unique sets of macromolecules, including type IV collagen, laminin, heparan sulfate (HS), proteoglycan and fibronectin, the successful
20 penetration of a basement membrane barrier probably requires the active participation of more than one tumor cell-associated enzyme.

 Due to its unique physical and chemical properties such as its polyanionic character and barrier properties
25 against macromolecules (Kanwar et al., 1980 J. Cell. Biol. V 86, pp 688-693), heparan sulfate (HS) is an important structural component of basement membranes. HS binds to fibronectin, laminin and type IV collagen, and these molecules have been collectively observed in the
30 basal lamina using antibodies raised against each component. HS may be involved in basal lamina matrix assembly by promoting the interactions of collagenous and non-collagenous protein components while protecting them against proteolytic attack. Thus, the destruction of HS
35 proteoglycan barrier could be important in basement membrane invasion by tumor cells.

The interactions between malignant cells and vascular endothelium have been studied using monolayers of cultured vascular endothelial cells that synthesize an extracellular matrix resembling a basement membrane. With this model, it has been found that metastatic B16 melanoma cells degrade matrix glycoproteins, such as fibronectin, and matrix sulfated glycosaminoglycans, such as heparan sulfate. Since heparan sulfate was released in solution as fragments approximately one-third their original size, it has been proposed that metastatic tumor cells characteristically have a heparan sulfate endoglycosidase.

The relation between metastatic properties and the ability of five B16 melanoma sublines of various implantation and invasion characteristics to enzymatically degrade subendothelial extracellular matrix indicated that highly invasive and metastatic B16 sublines degraded sulfated glycosaminoglycans faster than did sublines of lower metastatic potential (Nakajima et al., (1983), Science V 220, p 611), and intact B16 cells (or their cell-free homogenates) with a high potential for lung colonization also degraded purified heparan sulfate at higher rates than did B16 cells with a poor potential for lung colonization (ibid). The abilities of B16 cells to degrade HS from various origins and other purified glycosaminoglycans (heparin, chondroitin 4-sulfate, chondroitin 6-sulfate, dermatan sulfate, keratan sulfate, and hyaluronic acid) has been studied. In order to analyze glycosaminoglycan degradation products, an analytic procedure was developed using high-speed gel permeation chromatography (Irimura et al., (1983) Anal. Biochem. V 130, p 161; Nakajima et al., (1984) J. Biol. Chem. V 259, p 2283). HS metabolically labeled with [³⁵S]sulfate was purified from basement membrane producing

EHS sarcoma and PYS-2 carcinoma cells, and subendothelial matrices of bovine aortic endothelial (BAE) and corneal endothelial (BCE) cells (ibid). HS molecules purified from bovine lung and other glycosaminoglycans were
5 labeled with tritium at their reducing termini using NaB[³H]. These labeled glycosaminoglycans were incubated with B16 cell extracts in the absence or presence of D-saccharic acid 1,4-lactone, a potent α -glucuronidase inhibitor, and degradation fragments were analyzed by
10 high-speed gel permeation chromatography. HS isolated from the various origins described above were all degraded into fragments of characteristic molecular weight, in contrast to hyaluronic acid, chondroitin 6-sulfate, chondroitin 4-sulfate, dermatan sulfate, keratan
15 sulfate, and heparin, which were essentially undegraded. Heparin, but not other glycosaminoglycans, inhibited HS degradation. The time dependence of HS degradation into particular molecular weight fragments indicated that melanoma heparanase cleaves HS at specific intrachain
20 sites (ibid). In order to determine specific HS cleavage points, the newly formed reducing termini of HS fragments were investigated by: labeling with NaB[³H]; hydrolysis to monosaccharides; and analysis of these saccharides by paper chromatography. Since ³H-reduced terminal mono-
25 saccharides from HS fragments were overwhelmingly (>90%) L-gulonic acid, the HS-degrading enzyme responsible was an endoglucuronidase (heparanase).

HS-degrading endoglucuronidases have been found in
30 various tissues, such as human skin fibroblasts, rat liver cells, human placenta, and human platelets. HS-degrading endoglucuronidases in mammalian cells were reported previously by other investigators to be
"heparitinases" to indicate heparitin sulfate (heparan
35 sulfate)-specific endoglycosidase. However, heparitinase originally was used to designate an elimination enzyme

(EC 4.2.2.8) in Flavobacterium heparinum, and this enzyme cleaves nonsulfate and monosulfated 2-acetoamido-2-deoxy-alpha-D-glucosyl-D-hexuronic acid linkages of HS. Since HS-specific endoglycosidases in mammalian cells are endoglycuronidases, except for one found in skin fibroblasts, it was proposed that mammalian cell endoglucuronidases, capable of degrading HS should be called "heparanases", consistent with the currently used term "heparan sulfate".

Glycosaminoglycan endoglycosidases have been assayed for enzyme activity by some other means. For example, Oldberg et al. (1980, Biochem. V 19, pp 5755-5762) described an assay for a platelet endoglycosidase which degraded heparin-like polysaccharide. This assay involved measuring a decreasing amount of ³H-heparan sulfate, the decrease being a function of endoglycosidase activity.

Endoglycosidase assays using solid-phase substrates were described by Iverius (1971, Biochem. J. V 124, pp 677-683) and Oosta et al. (1982, J. Biol. Chem. V 257, pp 11249-11255). Iverius coupled a variety of glycosaminoglycans to cyanogen bromide-activated Sepharose 4B beads. In one case the endoglycosidase hyaluronidase was assayed for enzymic activity by incubation of the enzyme with chondroitin sulfate bound to Sepharose 4B. The enzyme activity was monitored by following the production of soluble uronic acid with a colorimetric assay procedure. Oosta et al. described an assay for heparitinase, an endoglycosidase from platelets which cleaves heparin and heparan sulfate.

The Oosta et al. system and assay comprised:

- (1) Coupling heparin with N-succinimide 3-(4-

hydroxylphenyl) propionate.

- (2) Labeling the coupled heparin by incubation with Na^{125}I and chloramine-T.
- 5 (3) Coupling the ^{125}I heparin to cyanogen bromide-activated beads of Sepharose 4B, and
- 10 (4) Incubating the endoglycosidase with the ^{125}I -heparin coupled to Sepharose 4B beads and measuring solubilized radioactivity.

In these two methods, glycosaminoglycans were cross-linked to agarose by the reaction of free amino groups of glycosaminoglycans and amino-reactive cyanogen bromide-activated agarose. Since glycosaminoglycans, such as heparin and heparan sulfate, have several free glucosamine amino groups, this type of crosslinking results in excessive covalent linkages between substrate molecules and agarose gel, resulting in a loss of susceptibility to endoglycosidases and nonlinear rates of degradation. Thus the most desirable solid-phase substrate for glycosaminoglycan endoglycosidase is glycosaminoglycan crosslinked to a solid support at one end of the molecule such as reducing terminal.

Heparanase, an endo- β -D-glucuronidase, has been associated with melanoma metastasis. Polyclonal antibodies directed against the murine N-terminal heparanase peptide detected a M_r -97,000 protein on SDS-polyacrylamide gel electrophoresis of mouse melanoma and human melanoma cell lysates. In an indirect immunocytochemical study, human A375-SM and mouse B16-BL6 melanoma cells were stained with the anti-heparanase antibodies. Heparanase antigen was localized in the cytoplasm of permeabilized melanoma cells as well as at

the cell surface of unpermeabilized cells.

Immunohistochemical staining of frozen sections from syngeneic mouse lungs containing micrometastases of B16-BL6 melanoma demonstrated heparanase localized in metastatic melanoma cells. Similar studies using frozen sections of malignant melanomas sected from patients indicated that heparanase is localized in invading melanoma cells. Studies relating to the present invention suggest that (a) the N-terminus of the heparanase molecule in mouse and human is antigenically related; (b) heparanase antigens are localized at the cell surface and in the cytoplasm of metastatic human and mouse melanoma cells; and (c) heparanase antigens are enriched in invasive and metastatic murine and human melanomas in vivo. Although polyclonal antibody preparations have been used herein, it is understood that monoclonal antibodies or a mixture of monoclonal antibodies with similar specificity may be also produced by well-known means and used analogously as desired.

20

A solid phase substrate which yields soluble labeled products upon hydrolysis by a glycosaminoglycan endoglycosidase and methods of producing said substrate are comprised in the present invention. The solid phase substrate comprises glycosaminoglycan bearing labeled N-acetyl groups and being reductively aminated at its reducing terminal end to produce an amine-terminus. The substrate is further coupled to an amino-reactive solid matrix through its amine-terminus.

30

A method of producing the solid phase substrate comprises the steps of: at least partially N-desulfating or N-deacetylating a glycosaminoglycan; labeling at least partially N-deacetylated or N-desulfated glycosaminoglycan to produce labeled glycosaminoglycan; completely N-acylating the labeled glycosaminoglycan with acyl

35

anhydride or acyl halide; reductively aminating a
reducing terminal end of said labeled glycosaminoglycan
to produce labeled amine-terminal glycosaminoglycan; and
coupling, through its terminal amine, the labeled amine-
5 terminal glycosaminoglycan to an amine-reactive solid
phase support to produce the solid matrix substrate.

The labeling may be accomplished by substitution on
amino groups of the partially N-desulfated or N-
10 deacetylated glycosaminoglycan of a substance yielding a
detectable signal. This substance may be a radioisotopic
label, a fluorescent label or an enzymatic label. A
fluorescent label is preferred for ease of assay and a
radioisotopic label for similarity to the natural
15 glycosaminoglycan.

Fig. 1. - Synthesis of a solid-phase heparanase
substrate: Chemical modification and radiolabeling of HS
and its coupling to amino-reactive agarose gel bead.
20

Fig. 2. - Elution profiles on high-speed gel
permeation chromatography of unmodified- and chemically
modified-HS before and after treatment with B16 melanoma
heparanase. HS, heparan sulfate; NDS-HS, N-desulfated
25 heparan sulfate NDS-HAc-HS, N-desulfated N-acetylated
heparan sulfate. These glycans labeled with tritium at
the reducing termini (open circles) and their fragments
produced by the incubation with B16 melanoma-cell
extracts in the presence of saccharic acid 1,4-lactone
30 (SAL) (solid circles), were chromatographed on two
sequential 0.7 x 75-cm columns of Fractogel-TSK HW-55(S)
with 0.2 M sodium chloride at a flow rate of 1.0 ml/min
at 55°C. Arrows (a)-(e) indicate the elution positions
of the standard glycans: (a) C6S from shark cartilage (M_r
35 60,000); (b) HS from bovine lung (M_r 34,000); (c)
heparin from porcine mucosal tissue (M_r 11,000); (d)

monosialosyl biantennary complex-type glycopeptide from porcine thyroglobulin (M_r 2190); (e) N-acetyl-D-glucosamine (M_r 221).

5 Fig. 3. - Dose dependent degradation of partially N-desulfated N[14 C]acetylated heparan sulfate (PNDS-N[14 C]Ac-HS) immobilized on agarose by B16 melanoma cell heparanase. The PNDS-N[14 C]Ac-HS (4500 cpm) immobilized on agarose was incubated with various amount of B16 cell
10 extract for 6 h (open squares), 12 h (open circles), and 24 h (open hexagons), or with various amounts of heat inactivated (100°C , 5 min) B16 cell extract for 12 hr (open triangles) in the presence of SAL. The released
15 radioactivity in a half volume of the supernatant versus the amount of cell extract added (ug protein) was plotted.

 Fig. 4 shows the levels of heparanase activity in the sera of controls and patients with malignant
20 melanoma.

 Fig. 5 shows levels of heparanase activity in sera of rats injected with a highly metastatic adenocarcinoma.

25 Fig. 6 shows the relationship between serum heparanase activity and the size of a primary metastatic tumor in rats.

 Fig. 7 shows the relationship between rat serum heparanase levels and numbers of metastases from a
30 malignant tumor.

 Fig. 8 shows the position of substrate hydrolysis for melanoma heparanase.

35

 Figure 9 - Autoradiograph of a 7.5% SDS-

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polyacrylamide gel containing proteins immunoprecipitated from human A375-SM (a-d) and mouse B16-BL6 (e-h) melanoma cell lysates metabolically labeled with [³⁵S]-methionine. Cell lysates were incubated with PBS (d,h), biotinylated
5 anti-heparanase antibodies (b,c,f,g), or biotinylated anti-heparanase antibodies preincubated with heparanase-peptide (a,e). They were subsequently incubated with streptavidin-agarose (a-h) and samples were run in the presence of β-mercaptoethanol. The molecular weight
10 markers at the far right are expressed in kDa.

Figure 10 - Micrographs of the immunocytochemical localization of heparanase in human A375-SM melanoma cells. Cultured cells were fixed (a-f), or permeabilized
15 (a,c-f). They were then incubated with anti-heparanase antibodies (a,b,e), with anti-heparanase antibodies preabsorbed with heparanase peptide (d) or with PBS (c,f). The cells were then washed and incubated with peroxidase-conjugated goat-anti-rabbit IgG (a,b,d,f) or
20 PBS (c,e). Original magnification x25; bar=30 μm. The micrographs are of the same magnification.

Figure 11 - Immunohistochemical localization of heparanase in a human cutaneous melanoma (a,b,c) or
25 murine melanoma metastasis in the lung (d,e,f). Frozen sections fixed with 2% paraformaldehyde were stained with hematoxylin and eosin (a,d) or incubated with anti-heparanase antibodies (c,f) or anti-heparanase antibodies preabsorbed by heparanase peptide (b) or PBS (e). The
30 sections were then incubated with goat-anti-rabbit IgG conjugated with peroxidase (b,c,e,f). Original Magnification x80; bar=20 μm. The micrographs are of the same magnification.

35 Immunoassays, such as those using antibodies raised to an glycosaminoglycan endoglycosidase such as heparan

sulfate endoglycosidase, for example, are described, which measure the enzyme. The present invention also describes a new assay for glycosaminoglycan endoglycosidase activity, most preferably that of the heparan sulfate endoglycosidase termed "heparanase". This new assay describes using a solid phase substrate which yields soluble labeled products upon hydrolysis by a glycosaminoglycan endoglycosidase. The new assay also describes novel adaptations of this solid phase enzymatic assay to liquid-phase conditions.

Among its many functions, the extracellular matrix is a tissue barrier that tumor cells must penetrate in the process of tumor invasion and metastasis. Extracellular matrix includes basement membranes and connective tissue stroma and is composed of collagens, proteoglycans, laminin, fibronectin and other glycoproteins. Degradation of this matrix, an important step in tumor cell invasion and metastasis, is mediated by a variety of degradative enzymes, including proteases and glycosidases of tumor or host cell origin (Mullins et al., *Biochim. Biophys. Acta*, 695, 177-214 (1983); Nakajima et al., *J. Cell. Biochem.*, 36, 157-167 (1988); Nicolson, *Biochim. Biophys. Acta*, 695, 113-176 (1982); Liotta, *Cancer Res.*, 46, 1-7 (1986); Sloane et al., *Cancer Met. Rev.*, 3, 249-263 (1984). One extracellular matrix-degrading enzyme, heparanase, was identified originally in murine and human melanoma cells (Kramer et al., *J. Biol. Chem.*, 257, 2678-2686 (1982); Nakajima et al., *Science*, 220, 611-613 (1983). The enzyme was purified and characterized as a glycoprotein of M_r 97,000 (Nakajima et al. *J. Cell. Biochem.*, 36, 157-167 (1988). Levels of heparanase activity have been directly correlated with the lung-colonizing potentials of murine metastatic melanoma cells (Nakajima et al. *Science*, 220, 611-613 (1983) and human melanoma cells (Nakajima et al.

Cancer Lett., 31, 277-283 (1986a), and such activity was found in sera of melanoma patients with metastatic disease (Nakajima et al. *J. Cell. Biochem.*, 36, 157-167 (1988). Structural analogues of heparanase substrate, such as
5 heparin and its chemically modified derivatives, inhibited B16 murine melanoma pulmonary metastases in experimental metastasis assays (Irimura et al., *Biochemistry*, 25, 5322-5328 (1986); Villanueva et al., 1988). Therefore, tumor cell heparanase is thought to
10 play an important role in melanoma invasion and metastasis.

Although the role of degradative enzymes in the process of tumor invasion and metastasis has been
15 investigated (Mullins et al. *Biochim. Biophys. Acta*, 695, 177-214 (1983); Liotta et al., *Cancer Res.*, 46, 1-7 (1986); Nicolson, *Biochim. Biophys. Acta*, 695, 113-176 (1982), the localization and sources of these enzymes have received little attention. 1982), the localization
20 and sources of these enzymes have received little attention. Current information on this is largely based on studies of proteases (Moscattelli et al., *Biochim. Biophys. Acta*, 948, 67-85 (1988), such as cathepsin B (Sloane et al., *Cancer Met. Rev.*, 3, 249-263 (1984) and
25 type IV collagenase (Liotta et al., *Nature (Lond.)* 284, 67-68 (1980). Using biochemical analyses of enzymatic activities in subcellular fractions, these enzymes have been associated with tumor cell surface membranes and various cellular compartments. Cathepsin B activities
30 were detected in lysosomal and plasma membrane fractions of B16 murine melanoma cells (Keren et al., *Cancer Res.*, 48, 1416-1421 (1988); Rozhin et al., *Cancer Res.*, 47, 6620-6628 (1987), and the enzyme was also localized at the cell surfaces of virus-transformed fibroblasts and
35 other cells (Sylvén et al., *Virchows Arch. B Cell Pathol.*, 17, 97-112 (1974). Heparanase activities have

been found on melanoma cell surfaces, in cell homogenates and shedded vesicles of melanoma cells (Nakajima et al. *Science*, 220, 611-613 (1983). Immunochemical studies on degradative enzymes have provided additional information on their localization and are particularly useful in identifying the source of an enzyme in tumors that also contain normal host cells and structures such as fibroblasts and lymphocytes. Cathepsin B was localized on some normal cellular components at the invasion front of rabbit carcinoma (Graf et al., *Lab. Invest.*, 45, 587-596 (1981).

In accordance with the present invention, anti-heparanase antibodies directed against the N-terminal heparanase peptides were prepared and characterized. Using an indirect immunochemical method, heparanase was localized at the cell surfaces and in the cytoplasm of human and mouse melanoma cells. The antibodies strongly stained metastatic melanoma nodules in a murine experimental metastasis model and human melanomas from patients with metastatic disease.

This solid phase substrate comprises a glycosaminoglycan bearing radioisotopically labeled N-acyl groups. These labeled N-acyl groups are preferably ³H labeled or ¹⁴C labeled acetyl groups although other labeled acyl groups, such as formyl or propionyl groups may be used. The solid phase substrate of the present invention may comprise as the glycosaminoglycan: hyaluronic acid, chondroitin 4-sulfate, chondroitin 6-sulfate, dermatan sulfate, keratan sulfate, heparan sulfate, heparin, or combinations thereof. The use of particular glycosaminoglycans will allow assays for the enzymic activity of endoglycosidases having a substrate specificity for the particular glycosaminoglycan being used.

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The amino-reactive solid matrix to which the amine-terminal labeled glycosaminoglycan may be bound may have many acceptable forms, both in the basic nature of the matrix and in the amine-reactive chemical site.

5

A preferable solid matrix is agarose-based, most preferably Sepharose or Sepharose derivatives in bead form (Pharmacia). Other solid matrices such as celluloses or polyacrylamides may be used provided that
10 the have amine-reactive substituent functions for coupling.

It is well known that Sepharose beads may be activated with cyanogen bromide and then coupled to amine-bearing molecules such as heparin, other glycosaminoglycans or glycosaminoglycan derivatives. Cyanogen
15 bromide mediated coupling, is a usable coupling method for practice of the present invention. Cyanogen bromide activated agarose or any other amine-reactive solid matrix may couple to more than one amine group of a
20 glycosaminoglycan and glycosaminoglycan derivative with multiple amine functions. This multiple coupling to labeled glycosaminoglycans could lead to insensitive and/or inaccurate glycosaminoglycan endoglycosidase
25 assays, since a single glycosidase-mediated hydrolytic event may not result in a soluble product, i.e., a product not linked to the solid matrix. Thus for the practice of the present invention, it is of importance that the labeled glycosaminoglycan derivative to be bound
30 to a solid matrix has but a single primary amino-group.

While a variety of amine-reactive substituents are known to those skilled in the art, an N-hydroxy succinide ester is a preferable amine-reactive function bound to a
35 solid matrix and is commercially available or readily synthesized. Such N-hydroxysuccinide esters couple to

- primary amine groups at a pH between about 6 and 9. Agarose may be activated by periodate oxidation to contain aldehyde functions. This aldehydic agarose may be reacted with labeled amine-terminal glycosaminoglycan and
- 5 the linkage stabilized by reduction with sodium cyanoborohydride. (Perikh et al., Methods in Enzym. Vol XXXIV, p 81 Acad. Press (1974)). Other commonly used procedures which may be used to link amine-bearing labeled glycosaminoglycans to solid matrices include:
- 10 using a carbodiimide and a carboxyl-bearing solid matrix; directly reacting the amine-bearing labeled glycosaminoglycan with a solid matrix bearing a bromoacetyl, diazonium or epoxy function.
- 15 The glycosaminoglycans generally have their amine functions either sulfated or acetylated. After at least partial N-desulfation or N-deacetylation, for example, the resultant primary amino groups on the glycosaminoglycan are available for labelling.
- 20 Deacetylation may be accomplished by hydrazinolysis under conditions avoiding excessive alkalinity which could lead to hydrolysis of glucosaminyl linkages. Desulfation may be accomplished by formation of pyridinium salts of the glycosaminoglycan followed by solvolysis in
- 25 dimethylsulfoxide. Amino group labeling is accomplished by reaction with a fluorescent compound such as fluorescein isothiocyanate, an enzyme such as alkaline phosphatase (and a bifunctional coupling agent) or with a radioisotopically labeled acyl anhydride or acyl halide.
- 30 A label is then covalently attached to at least some of the free amine groups. Remaining free amine groups of the labeled glycosaminoglycan are then acylated, for example by acetic anhydride treatment. The acylated labeled glycosaminoglycan is then aminated at its
- 35 reducing terminal. This amination is accomplished by incubation with an amine salt to form a Schiff base with

the terminal and subsequent reduction to form a terminal amine.

Amino group labeling may be accomplished by coupling
5 a measurable compound or active protein to at least a few
of the amino groups. The measurable compound may be one
of the many known to be highly absorbent of visible light
or more preferably one which is fluorescent when excited
by irradiation at particular wavelengths such as
10 fluorescein as mentioned above. Labeling by attachment
of enzymes (as alkaline phosphatase mentioned above) as
active proteins to the partially N-desulfated or N-
deacetylated glycosaminoglycan is also a possibility.
Labeling by enzymes or measurable compounds having light
15 absorbent or fluorescent structures, however, may involve
sterically bulky substituents. Such sterically bulky
substituents may, when substituted at too high a level,
render glycosaminoglycan derivatives which are poor
substrates for glycosaminoglycan endoglycosidases. In
20 preliminary experiments, a partially N-desulfated heparan
sulfate was coupled in a 1:1 ratio to fluorescein
isothiocyanate. This fluorescein labeled derivative was
found to be a good substrate for melanoma heparanase. It
is contemplated that up to a 10:1 ratio of fluorescein to
25 HS may be produced and serve as a heparanase substrate.

One or a very few of these bulky substituents may
not hinder substrate activity and result in good labeled
substrates. Another potential problem with enzyme labels
30 is that enzymes generally contain free amine groups which
may bind to amine-reactive solid matrices. One preferred
label for glycosaminoglycans is a radioisotopic label
similar or identical in structure to naturally occurring
N-substituents. While ³⁵S-sulfate N-substituents could be
35 utilized, ¹⁴C- or ³H-acetyl N-substituents are preferred
as readily produced. Although the subsequently described

substrates and procedures relate primarily to radioisotopic labeling there are largely applicable in principle to other labels, particularly fluorescent labels.

5 When N-radioisotopically labeled glycosaminoglycans are attached by a single bound at one end to a solid matrix, a solid phase substrate for a glycosaminoglycan endoglycosidase is created. As described elsewhere herein, this solid phase substrate yields soluble radio-
10 isotopically labeled substances as a function of glycosaminoglycan endoglycosidase enzymatic activity. An alternative manner of measuring this same activity would be to observe the disappearance of radioisotopic label bound to the solid matrix as a function of enzymatic
15 activity. This type of measurement has the disadvantage of being a negative measurement and also that incubation supernatant would have to be carefully removed from residual solid matrix substrate.

20 In a broad sense, the solid phase substrate of the present invention is one which yields soluble products labeled with a detectable signal upon hydrolysis by a glycosaminoglycan endoglycosidase. This solid phase substrate comprises a glycosaminoglycan bearing a label
25 which does not prevent hydrolysis of the labeled glycosaminoglycan by a glycosaminoglycan endoglycosidase. The labeled glycosaminoglycan is linked through a single end, preferably the reducing terminal end and by a single covalent linkage, to a solid matrix. The detectable
30 signal may be radioisotopic, light absorbent, fluorescent or enzymatically active. The solid matrix is preferably hydrophilic and may include polymers such as cellulose, dextran, polyacrylates or their derivatives, alone or in combination. The substrate of the present invention may
35 be soluble if a detectable label is present along with a tagging molecule. The tagging molecule may be used as a

'handle' for removal of a portion of attached glycosaminoglycan.

5 The labeling of at least partially desulfated or
deacylated glycosaminoglycan is most preferably accomplished by treatment with ³H-acetic anhydride or ¹⁴C-acetic anhydride, although analogous acetyl halides, particularly chlorides or also alkyl bromides are contemplated as useful. In addition to other acyl functions such as formyl or propionyl, other coupling methods
10 may be used in this labelling procedure.

 The substrate of this invention may also be a liquid phase substrate with separation of the cleaved products
15 from the uncleaved substrate occurring after the enzymatic reaction. In this scheme, a glycosaminoglycan such as heparan sulfate, for example, could be tagged at one end, preferably the reducing end, to another molecule. The glycosaminoglycan should be labeled at
20 additional sites by other molecules such as ¹²⁵I, fluorescein, enzymes, and the like, that may be used for detection of cleaved products in the assay. Among the advantages available with a liquid substrate of the type described herein should be an assay with increased
25 sensitivity to the action of glycosaminoglycan endoglycosidases. This increased sensitivity would at least in part relate to an enhanced availability in solution to soluble enzymes.

30 The molecular tag at one end of the glycosaminoglycan could be either a small molecule, such as fluorescein or biotin, or a larger molecule, such as a peptide or a protein. The linkage of this molecule to an end of the glycosaminoglycan substrate must not
35 significantly inhibit the hydrolysis of the tagged glycosaminoglycan by the glycosaminoglycan

endoglycosidase. The molecular tag should have the ability to act as a potential 'handle' for the labelled glycosaminoglycan chain and for the residue of the glycosaminoglycan chain remaining after cleavage by a glycosaminoglycan endoglycosidase. As a 'handle', the molecule would be able to act as a point of attachment for a protein molecule having affinity for the bound tagging molecule. Such a protein-molecule relationship will enable tagged portions of the labeled glycosaminoglycan to be readily separated from labeled but untagged portions liberated by endoglycosidase-induced hydrolysis of glycosaminoglycan substrate hydrolysis. The molecular tag should be either: a) a haptenic molecule capable of generating specifically binding antibodies when attached to a carrier such as a protein and immunogenically administered to an animal; b) a segment of or a whole immunogenic substance such as a protein or peptide; or c) a substance having a high binding affinity for existent proteinaceous molecules such as avidin or protein A, for example.

Following incubation with samples containing endoglycosidase activity, the uncleaved products may then be separated from the cleaved products by incubation with, for example, solid-phase antibodies having an affinity for the tag. Proteins other than antibodies that bind the molecular tag that has been attached to the end of the glycosaminoglycan may also be used to separate uncleaved glycosaminoglycan. If solid phase antibodies or solid phase binding proteins are used, the solid phase may be any support that can be readily coupled or absorbed to antibodies or binding proteins and that can affect a separation of cleaved product from uncleaved substrate. Commonly-used examples of solid phase include agarose; Sepharose; polymers, such as polystyrene; glass; cellulose and glass beads; and magnetizable beads. The

solid-phase could be in the form of large or small particles or a tube or microtiter plate or other device that is readily adaptable to the detection system.

5 The separation of cleaved from uncleaved glycosaminoglycan products can also be achieved by an immunoprecipitation reaction that does not require antibodies to be linked to a solid phase (see Morgan et al. (1962) Proc. Soc. of Exp. Biol. Med., V 110, pp 29-
10 35). The precipitating antibodies could be directed toward the molecule tagged at the end of the glycosaminoglycan chain.

 To facilitate binding at one end to a solid matrix,
15 a further modification of labeled glycosaminoglycan was needed. This modification involved the placement of a primary amino group at one end of the labeled glycosaminoglycan. A preferable method of accomplishing this placement was to incubate the labeled
20 glycosaminoglycan with an ammonium salt and sodium cyanoborohydride at an alkaline pH. A Schiff base initially forms between ammonia and the aldehydic carbonyl group of the terminal hexose. This Schiff base is reduced to a primary amine by the sodium
25 cyanoborohydride.

 The synthetic steps to produce the solid phase substrate of the present invention generally include partial N-deacylating, for example, by hydrazinolysis, or
30 N-desulfating, for example by solvolysis in dimethylsulfoxide; an N-acylating step with labeled acyl anhydride or halide for radioisotopic labeling; a reductive amination step; and coupling to a solid matrix through the newly introduced terminal amine. An often
35 preferred final step, to insure that no amine-reactive functions remain on the solid matrix, is to incubate the

product of the matrix -labeled amine-terminal glycos-
aminoglycan coupling with sodium cyanoborohydride and a
compound bearing a free amino group. This latter
compound may, for example, be one such as ethanolamine or
5 glycine ethyl ester.

The substrates and procedures of the present
invention present numerous advantages for the assay of
glycosaminoglycan endoglycosidase enzymic activity. For
10 example, the substrate of the present invention is bound
to a solid matrix via a single carbohydrate-bound amino
ligand and yields a linear pattern of enzymatic products.

In the past, proteoglycans containing glycosamino-
15 glycans as well as a bound protein component have been
bound to a solid matrix of cyanogen bromide-activated
agarose. The proteoglycan was thereby likely bound to
the agarose primarily through its proteinaceous com-
ponent. Thus, both proteolytic as well as glycosamino-
20 glycan endoglycosidic activity may liberate a soluble
product. The specificity of the assay for enzymic
activity of the endoglycosidases is less than the results
shown with the present invention.

25 A heparanase (heparan sulfate endoglycosidase)
obtained from a human melanoma cell line was found to
only partially degrade N-desulfated, N-acetylated
heparin. This same enzyme preparation was found to
efficiently cleave N-desulfated heparan sulfate as well
30 as N-desulfated N-acetylated heparan sulfate into
characteristic degradation fragments.

While there are many glycosaminoglycan endoglycosi-
dases, heparan sulfate endoglycosidase or heparanase, the
35 endoglycosidase utilizing heparan sulfate as a preferred
substrate, was chosen as a typical example to demonstrate

a preferred embodiment of the present invention. Additionally, an N-hydroxy succinide agarose derivative was selected as a preferred solid matrix to couple labeled amine-terminal heparan sulfate to produce a solid phase substrate. Heparanase activity produced soluble radioisotopically labeled products as demonstrated specifically in many of the following examples.

Melanoma heparanase is an endo- β -glucuronidase which specifically cleaves HS at intrachain sites. Such melanoma heparanase specificity is illustrated in Figure 8. Thus, the separation of the reaction products from the substrates based on their size is required for the heparanase assay. Although previously established methods such as polyacrylamide gel electrophoresis and high-speed gel permeation chromatography are useful for the characterization of degradation fragments, they are not suitable for rapid and microscale quantitative assays of large sample numbers. To perform rapid quantitative assays, a covalently linked substrate is required. The presently developed solid phase assay substrate is partially N-deacetylated or N-desulfated, N-[^3H or ^{14}C]-acetylated HS coupled with Affi-Gel 15. In this substrate a HS derivative is linked to agarose through only one covalent bond (Figure 1). This product is one of the most sensitive endoglycosidase substrates to be developed. This substrate has now been successfully used for mouse and human melanoma heparanase assays. The same type of derivative has also been produced by using Reacti-Gel (NW-65F) (Pierce, Rockford, IL). However, both Affi-Gel 15 and Reacti-Gel (HW-65F) use quite large particles and these retain significant amounts of high molecular weight materials in the gel matrices. This may be a problem in some quantitative heparanase assays, therefore, we developed a more desirable assay substrate by using Affi-Gel 701 or 702 (Bio-Rad) which are

approximately 1-3 microns in diameter with an exclusion limit of M_r 10,000. The specific synthetic procedure was as follows. Radiolabeled HS was reduced with sodium borohydride to form a sugar alcohol at the reducing terminal. The sugar alcohol was converted to a primary aldehyde by periodate oxidization. This aldehyde group was then linked to amino-derivatized beads, such as Affi-Gel 701, through a Schiff base and stabilized by reduction with sodium cyanoborohydride. Yet further proposed procedures, similar to our previously developed methods, are contemplated as useful. Radiolabeled HS whose amino groups have been sulfated or acetylated should be aminated at the reducing terminal with ammonia under reducing conditions. Affi-Gel 702 should be converted to an amino-reactive bead by derivatization with *N*-hydroxysuccinimide or *N,N'*-carbonyldiimidazole, and then the aminated radioactive HS should be linked to amino-reactive Affi-Gel 702. The substrate may be made more radioactive by use of ^{125}I -labeled HS, although iodination of HS with Bolton and Hunter Reagent may be disadvantageous because of potential structural change. On the other hand, the assay may also be improved by use of fluorescein-labeled HS for routine clinical studies, and fluorescein-labeled HS is suitable for a rapid analysis of degradation fragments on HPLC equipped with a flow fluorescence detector.

The assay measuring levels of a glycosaminoglycan endoglycosidase such as heparan sulfate endoglycosidase (heparanase) may also be performed in an immunoassay format using polyclonal and/or monoclonal antibodies raised to the endoglycosidase. Preferably, antibodies with relatively low cross-reactivity to other endoglycosidases, such as the platelet endoglycosidase described by Oldberg, et al. (1980) *Biochem.*, V 19, pp 5755-5762, can be used. The antibodies may be used with a variety

- of immunoassay techniques to measure the endoglycosidase protein directly. The endoglycosidase may be measured by either a radioimmunoassay described by Berson and Yalow (1968) Clin. Chem. Acta., V 22, p 51 or an immunoradio-
- 5 metric (IRMA) assay described by Miles, et al. (1976) Anal. Biochem., V 61, pp 209-224 using ¹²⁵I-labeled antigen or antibody. The endoglycosidase may also be measured by an enzyme immunoassay that uses either a competitive-binding assay or a "sandwich" assay analogous
- 10 to an IRMA and using alkaline phosphatase, horse radish peroxidase, or any other enzyme coupled to an antibody or to the endoglycosidase as reviewed by Wisdom (1976) Clin. Chem., V 22, pp 1243-1255.
- 15 The cellular localization of tumor invasion- and metastasis-associated degradative enzymes has been studied biochemically as well as immunochemically. However, most of our current knowledge on this subject is based on studies that utilized proteolytic enzymes, such
- 20 as cathepsin B and type IV collagenase. Sloane and her colleagues (Rozhin et al., *Cancer Res.*, 47, 6620-6628 (1987); Sloane et al., *Proc. Natl. Acad. Sci.*, 83, 2483-2487 (1986) reported that cathepsin B-like activity in metastatic B16 melanoma cells was detected in plasma
- 25 membrane-associated fractions. Keren et al., *Cancer Res.*, 48, 1416-1421 (1988) reported that cathepsin-B like activity was present in 2% butanol extracts containing cell surface associated molecules of metastatic B16 melanoma and fibrosarcoma cells, and the plasma membrane-
- 30 associated cathepsin B activity appeared to correlate with the metastatic potentials of the tumor cells, including melanoma, rhabdomyosarcoma, and mammary carcinoma cells. There is a good correlation between type IV collagenase activities in the conditioned media
- 35 of tumor cells and their invasiveness and metastatic potentials (Liotta et al., *Nature (Lond.)* 284, 67-68

(1980); Nakajima et al., *Cancer Res.*, 47, 4869-4876
(1987). Nakajima et al., *Cancer Res.*, 49, 1698-1706
(1989) found a plasma membrane associated 64 kDa type IV
collagenase on rat mammary adenocarcinoma cell surfaces.
5 Interestingly, in an immunohistochemical study with the
rabbit V2 carcinoma, Graf et al., *Lab. Invest.*, 45, 587-
596 (1981) observed that cathepsin B was localized to
normal cells, including fibroblasts and leukocytes, at
the tumor invasion front, but it was not found on the
10 carcinoma cells. Using indirect immunofluorescence
methods, Woolley et al., In: P. Strauli, A.J. Barret,
and A. Baici (eds), *Proteinases and tumor invasion*, pp
97, Raven Press, New York (1980) and In: L. S. Liotta
and I.R. Hart (eds), *The Tumor invasion and metastasis*,
15 pp 391, Martinus Nijhoff, the Hague (1982) studied the
localization of collagenase in human primary and
metastatic melanoma tissues. Three out of 5 primary
melanoma specimens and 6 of 15 metastasis specimens
examined had positive staining with the anti-collagenase
20 antibodies, but the results were too variable to draw any
conclusions. They found fluorescence in the connective
tissue surrounding melanoma nests in some cases and at
the junctions between tumor and normal tissues in others.
Data from previous observations of the present inventors
25 on heparanase activity in melanoma cells suggested that
heparanase is localized in the cytoplasm and at the cell
surface. Heparanase activity has been recovered from
intact melanoma cells and their homogenates (Nakajima et
al., *Science*, 220, 611-613 (1983), as well as from
30 their shedded plasma membrane vesicles. Other reports
have also suggested that heparanase activities are plasma
membrane-associated or secreted by malignant cells.
Heparanase activity has been observed in the serum-free
conditioned medium of metastatic ESb murine lymphoma
35 cells and also in intact, viable ESb cells incubated with
 [³⁵S]SO₄-labeled subendothelial extracellular matrix.

Vlodavsky et al., *Cancer Res.*, 43, 2704-2711 (1983) . In another study, Savion et al., *J. Cell. Physiol.*, 118, 169-178 (1984) reported that murine activated T lymphocytes and inflammatory peritoneal macrophages are able to release heparanase and degrade heparan sulfate in the subendothelial extracellular matrix. These results suggest cell surface association or cell secretion of heparanase. On the other, Ricoveri and Cappelletti *Cancer Res.*, 46, 3855-3861 (1986) suggested that the metastatic tumor cell heparanase is a lysosomal enzyme and scarcely released in vitro. However, the immunochemical localization of heparanase has not previously been attempted.

Melanoma heparanase, an endo- β -D-glucuronidase, is thought to play an important role in melanoma metastasis (Nakajima et al., *J. Cell. Biochem.*, 36, 157-167 (1988)). It is one of several tumor cell degradative enzymes that have been systematically studied. Heparanase activities have been found in human and mouse melanoma cell homogenates and their conditioned media as described above. The successful preparation of anti-heparanase enabled the study of the localization of murine and human heparanases in normal and tumor tissues. To ensure the specificity of the antibodies, extensive characterization was carried out. Immunoprecipitates of the antibodies with melanoma cell lysates were subjected to SDS-PAGE to determine the molecular weights of the proteins the antibodies recognized. The pIs of the immunoprecipitated proteins were also examined using 2-dimensional gels. In an attempt to aid the purification of heparanase, we also constructed anti-heparanase antibody-agarose affinity column using AffiGel 10 (Bio-Rad). Heparanase activity was only detected in the fractions eluted with citrate buffer at pH lower than 4. The finding that anti-murine heparanase antibodies cross-react with human heparanase

is consistent with our previous observations on human and mouse heparanase. Human and mouse heparanases share a number of biochemical characteristics, such as molecular weight, substrate and inhibitor specificity, and optimal pH for enzymatic activity. These observations suggest that heparanase molecules may be highly conserved between mouse and human.

The generation of anti-heparanase antibodies also gave us an opportunity to localize heparanase in melanoma cells and tissues. Heparanase antigen was localized predominantly in the cytoplasm of both B16-BL6 and A375-SM cells, as well as at the cell surface of these cells. It remains to be determined which fraction of heparanase antigen (cell surface, cytoplasmic or both) represents the active form of the enzyme. The relationship between cell surface, cytoplasmic, and secreted forms of heparanase, and the regulatory mechanisms involved are not clear. The immunocytochemical staining of B16-BL6 and A375-SM melanoma cells appeared to be heterogeneous, which is consistent with the notion of tumor cell heterogeneity in the expression of gene products.

Since heparanase activity has been correlated with the metastatic potentials of murine B16 melanoma cell lines (Nakajima et al., *J. Biol. Chem.*, 259, 2283-2290 (1984), heparanase antigen was examined in mouse and human melanoma tissues. Metastatic lung nodules in a murine experimental metastatic model were intensely stained with the anti-heparanase antibodies, although the background staining in normal pulmonary tissues surrounding the tumor was barely noticeable. The background in normal lung tissue may be due to the presence of the pulmonary alveolar macrophages in which heparanase activity was observed (Nakajima et al., *J. Cell. Biol.*, 101, 215a (1985); Savion et al., *J. Cell.*

Physiol., 118, 169-178 (1984). The present invention takes advantage of the fact that the anti-mouse-heparanase antibodies cross-reacted with human heparanase and allowed study of the immunochemical localization of heparanase antigen in human melanoma tissue. Melanoma tissues from patients with metastatic diseases were examined. Melanoma cells became stained with the antibody but the surrounding normal tissues were not except that the endothelial cells of an arteriole (Fig. 3c) were slightly stained. It is possible that endothelial cells may express heparanase along with other degradative enzymes in the process of neo-angiogenesis and wound healing. The data presented in accord with the present invention suggest that heparanase antigens in invasive melanoma cells are significantly enriched compared to surrounding normal tissues.

The localization of heparanase antigen at the melanoma cell surfaces supports the concept of cell surface involvement in tumor invasion and metastasis (Nicolson, *Biochim. Biophys. Acta*, 695, 113-176 (1982); Moscatelli et al., *Biochim. Biophys. Acta*, 948, 67-85 (1988). The localization of heparanase to invasive and metastatic melanoma cells in melanoma tissues strongly suggests that heparanase plays an important role in melanoma invasion and metastasis. Such localization may lead to changes in therapeutic strategy, more metastatically or invasion prone tumors being likely to contain more detectable heparanase and being worthy of more aggressive therapy, for example.

The endoglycosidase may also be measured in these assays by using fluorescein or other fluorescent compounds as reviewed by Gerson (1984) J. Clin. Immunoassay, V 7, pp 73-81, by chemiluminescence as reviewed by Weeks and Woodhead (1984), J. Clin

Immunoassay, V 7, pp 82-89, or by other labels. In all of these assays, the bound endoglycosidase may be separated from the unbound endoglycosidase by a variety of techniques. These include solid-phase immobilization of a primary (anti-endoglycosidase) antibody, avidin-biotin separation using a biotin-labeled antibody and solid phase avidin, "double antibody" precipitation, or by using solid phase antibody against a hapten like fluorescein coupled to a primary antibody, or by using a solid phase "second antibody". ("Double antibody" is defined as a heterologous antibody that binds the anti-endoglycosidase antibody as in Midley, et al. (1969) Acta Endocrinol., V 63, Supp. 142, p 247).

The solid phase systems mentioned above can include polymers, such as polystyrene; agarose; sepharose; cellulose; glass beads; and magnetizable particles of cellulose or other polymers. The solid-phase can be in the form of large or small beads or particles; tubes; plates; or other forms.

Kits useful in the present invention include those of the general type described by Szczesniak (U.S. Pat. No. 3,899,298). Such kits comprise a carrier being compartmentalized to receive at least one, or at least two or at least three or more containers and to maintain said containers in closed confinement. A first container may contain purified anti glycosaminoglycan endoglycosidase antibody (preferably monoclonal), either in solution, in freeze-dried form or covalently bound to the inside thereof, such as for example if such container is a test tube. A second container may then contain a second anti glycosaminoglycan endoglycosidase antibody (also preferably monoclonal). Alternatively, another container may contain detectably labeled glycosaminoglycan endoglycosidase antigen. At the time

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of testing for glycosaminoglycan endoglycosidase antigen in the sample, the sample is added to the first container containing the monoclonal antibody, incubated, and then antibody from the second container is added thereto to provide a "sandwich". The antibody in the second container may be detectably labeled as, for example, by a radiolabel or an enzyme label. Another container in the kit may contain appropriate enzyme substrate in order to carry out the "ELISA" methodology. Any number of variations or permutations consistent with the various techniques for use in the detection of glycosaminoglycan endoglycosidase antigen may be envisioned for the preparation of a kit. These are all matters of choice, determined by the ease of handling, rapidity and efficiency of the testing.

Quantitative analysis of glycosaminoglycan endoglycosidase antigen can be carried out by interpolation into a standard curve, as is known in the art. A multiplicity of container means, each one having a different amount of glycosaminoglycan endoglycosidase antigen can be present in the kit for such a purpose.

In still another embodiment, the antibody can be immobilized onto plastic strips which are then brought into contact with the samples suspected of containing glycosaminoglycan endoglycosidase antigen. Subsequently, the strip is contacted with a solution containing a second, enzyme labeled anti glycosaminoglycan endoglycosidase antibody; this results in a sandwich forming on the strip. Finally, introduction of the strip into a color developing solution (such as substrate for the enzyme) and detection of color, is a rapid efficient and inexpensive method for qualitatively, and even roughly quantitatively determining glycosaminoglycan endoglycosidase antigen in animal samples.

The immunoassays of the present invention may use antibodies which are very discriminating between the different glycosaminoglycan endoglycosidases, particularly for heparan sulfate endoglycosidase. The methodology described herein should be superior in sensitivity and ease to other known methods of glycosaminoglycan endoglycosidase detection.

In an analogous manner, kits are easily constructed comprising labeled glycosaminoglycan affixed, preferably through its amino-terminal end to a molecular tag as described above. Such a kit would also comprise a specific binding agent capable of removing tagged glycosaminoglycan or tagged fragments thereof from solution. The specific binding agent may already be bound to a solid matrix or may be so bound by the user of the kit and assay. Preferred binding agents are proteins, more preferably, antibodies and most preferably, monoclonal antibodies.

The development of specific diagnostic tests for infections of glycosaminoglycan endoglycosidase has become medically desirable for purposes such as detection of tumors. Such specific diagnostic tests as described herein may be developed through the use of monoclonal or polyclonal antibodies specifically binding to glycosaminoglycan endoglycosidase.

These examples are presented to describe preferred embodiments and utilities of the present invention and are not meant to limit the present invention unless otherwise specified in the claims appended hereto.

Purification of heparanase

Melanoma heparanase was purified from cultured

murine melanoma cells. Murine B16-F10 melanoma cells (Fidler, *Nature (London) New Biol.* 242, 148-149 (1973) were cultured in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F-12 nutrient mixture (DME/F12; Hazleton, Lenexa, KS) supplemented with 5% heat-inactivated fetal bovine serum (FBS; Hyclone, Logan, UT). Cells in a subconfluent culture were harvested by a brief treatment with 2 mM EDTA in phosphate buffered saline at pH 7.2 (PBS) and 2×10^8 cells were extracted at 4°C for 30 min in 50 ml of 50 mM Tris-HCl, pH 7.5 containing 1 mM phenylmethylsulfonyl fluoride (PMSF; sigma, St. Louis, MO), 5 mM N-ethylmaleimide (NEM; Sigma), 0.5% Triton X-100 and 0.05% sodium azide (buffer 1). The cell extract was centrifuged at 30,000 x g for 30 min at 4°C and the supernatant containing approximately 50 mg protein was passed through a column of heparin-Sepharose (Pharmacia LKB Biotechnology, Piscataway, NJ) equilibrated with buffer 1. The heparin-Sepharose column was sequentially washed with buffer 1, 20 mM sodium acetate, pH 6.0, containing 0.2% Triton X-100 (buffer 2), and 0.15 M sodium chloride, 20 mM sodium acetate, pH 6.0 (buffer 3). Heparin-binding proteins were eluted with a linear sodium chloride gradient (0.15 M - 1.2 M) in 20 mM sodium acetate, pH 6.0. The eluted materials were monitored by measuring absorbance at 280 nm, and the heparanase activity was measured using heparanase solid phase substrates as previously described (Nakajima et al. *Anal. Biochem.*, 157, 162-171 (1986b)). Heparanase active fractions contained approximately 6 mg proteins and 90% of total activity. After dialysis against buffer 3 the heparanase fraction was centrifuged at 30,000 x g for 30 min and the supernatant was loaded on a concanavalin A-Sepharose column (Pharmacia LKB) equilibrated with buffer 3. After washing the column with buffer 3, concanavalin A-binding proteins were eluted with 1.0 M α -methyl-D-mannopyranoside (Sigma) in buffer 3. The eluent

containing approximately 0.2 mg protein and 48% of total heparanase activity was collected and extensively dialyzed against buffer 3, and then passed through a N-acetylated N-desulfated heparin-Sepharose column

5 (Nakajima et al. *Anal. Biochem.*, 157, 162-171 (1986b)) equilibrated with the same buffer. After washing with 0.3 M sodium chloride, 20 mM sodium acetate, pH 6.0, binding proteins were eluted with 0.6 M sodium chloride 20 mM sodium acetate, pH 6.0. Highly purified heparanase

10 fractions (0.08 mg protein, 34% total activity) were dialyzed against 12.5 mM Tris-HCl, 0.15 M NaCl, pH 7.5, and concentrated with Centricon 30 concentrators (Amicon, Danvers, MA), and then subjected to high speed gel permeation chromatography using a Waters 600E system

15 equipped with a PROTEINPAK 300 SW column (Waters, Milford, MA). The proteins were eluted with 12.5 mM Tris-HCl, 0.15 M NaCl, pH 7.5 (1 ml/min, 23°C) and the elution was monitored at 280 nm with a Waters Model 490 multiple wavelength detector. The second peak fractions

20 of molecular weight range approximately 100,000 to 150,000 contained heparanase (0.04 mg protein, 29% of total activity). The heparanase fraction was further subjected to chromatofocusing using a PBE94 column (Pharmacia LKB). Starting buffer and elution buffer were

25 25 mM imidazole-HCl, pH 7.5 and Polybuffer 74-HCl (Pharmacia LKB), pH 4.0, respectively. Heparanase was eluted as a sharp peak at pH 5.0-5.2, and approximately 0.01 mg protein with 18% of total activity was recovered. The purified heparanase was analyzed by sodium dodecyl

30 sulfate (SDS)-polyacrylamide gel electrophoresis according to the methods by Laemmli *Nature (London.)* 227, 680-685, (1970) and its apparent molecular weight was determined as 97,000. The heparanase band was cut and electroeluted using an ISCO electrophoretic concentrator

35 Model 1750 (ISCO, Lincoln, NE) and its N-terminal amino acid sequence was analyzed by a model 470A gas-phase

sequencer (Applied Biosystem Inc.) with an attached model 120A-PTH analyzer.

Immunological reagents

- 5 Heparanase peptide (EEDLGKSREGSRTDD-C) was designed based on an analysis of the hydrophilicity of amino acid residues of the heparanase N-terminal sequence (EVDVDGTVEEDLLGKSREGSRTDD) and was synthesized on a DuPont solid phase peptide synthesizer according to the method by Merrifield *J. Am. Chem. Soc.*, 85, 2149-2154 (1963). The peptide was designed to have an additional cysteine residue at C-terminus to facilitate coupling to a carrier protein, keyhole limpet hemocyanin (KLH; Calbiochem, La Jolla, CA). Other protein carriers are of course usable in place of KLH. Coupling of the peptides with KLH was performed by the methods described by Liu et al., *Biochemistry*, 18, 690-697 (1979). Briefly, LKH was suspended in 10 mM sodium phosphate buffer pH 7.2 (PB) at 20 mg/ml and dialyzed using a cellulose membrane with a molecular weight cutoff of 12,000-14,000 (Spectrum, Los Angeles, CA) against 2 L of the same buffer overnight (3 changes). The dialysate was centrifuged at 10,000 x g for 10 min to remove undissolved material. The concentration of KLH was adjusted to 16 mg/ml after protein concentration was determined by the Pierce Coomassie protein assay (Pierce, Rockford, IL) using bovine serum albumin (Sigma) as a standard. The peptide was dissolved in PB at a concentration of 5 mg/ml, and m-maleimidobenzoyl-N-hydroxysuccinimide ester (MBS; Sigma) was dissolved in dimethylformamide (DMF; Aldrich, Milwaukee, WI) at a concentration of 6 mg/ml. The MBS solution was slowly added to the LKH solution (MBS:KLH = 0.51 mg:4.0 mg) and the mixture incubated at 25°C for 30 min with gentle stirring. The activated KLH was separated from remaining low molecular weight MBS by gel filtration chromatography. It appeared at the void
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volume of a Bio-Gel P-30 gel filtration column (2.5 x 25 cm) and was eluted with 50 mM phosphate buffer, pH 6.0. The peptide solution (5 mg/ml) was then mixed with the activated KLH, and the pH was adjusted with sodium hydroxide to 7.0-7.5. After incubation at 25°C for 3 h, the peptide-KLH mixture was centrifuged at 10,000 x g to remove undissolved material. The coupling efficiency was determined by the Ellman assay for free thiol (Ellman et al., *Arch. Biochem. Biophys.*, 82, 70-77 (1959). The coupling efficiency was greater than 78.5%. The KLH-coupled peptide antigens were aliquoted and stored at -80°C.

New Zealand White rabbits were immunized subcutaneously with KLH-coupled heparanase-peptide. Prior to injection, the antigen was emulsified with either complete Freund's adjuvant for the primary immunization or incomplete Freund's adjuvant for subsequent boosting immunizations. Dosage of antigen was 500 µg of peptide for the first injection and 250 µg for subsequent boosting injections. The interval between first and second injections was 3 weeks and boosting injections were administered when the antisera titer began to drop. Antibody activity to heparanase-peptide was determined using an enzyme-linked immunosorbent assay with peptide coated 96-well plates (Costar, Cambridge, MA). The plates were prepared by coating each well with 1 µg of synthetic peptide antigen in 100 µl of 0.1 M sodium bicarbonate, pH 9.0, and allowing the buffer to evaporate at 37°C overnight. The titers of antisera raised against heparanase peptides were between 1:800 and 1:1200 when preimmune sera were used as reference.

The antibodies directed against heparanase-peptide were further purified by antigen affinity chromatography using heparanase-peptide covalently coupled to Affi-Gel

10 beads (Bio-Rad, Richmond, CA). The sera were first precipitated with 45% (v/v) ammonium sulfate at 4°C and centrifuged at 10,000 x g for 20 min. The precipitates were then dialyzed against 1000 vol of 5 mM HEPES, 150 mM NaCl, pH 7.4, overnight at 4°C with 3 changes of buffer. The dialysates were loaded on Affi-Gel-heparanase-peptide affinity columns. The affinity columns were washed extensively with 5 mM HEPES, 150 mM NaCl, pH 7.4, and then eluted with 50 mM sodium citrate, 150 mM NaCl, pH 5.5, and finally with 50 mM sodium citrate, 150 mM NaCl, pH 2.0. The fractions were collected and protein concentrations determined by the Pierce Coomassie Protein Assay, using IgG as the protein standard. Antibody activity to heparanase-peptide was determined by ELISA assay. Antibody fractions eluted at pH 2.0 were collected and designated as anti-heparanase antibodies. The antibodies were biotinylated according to the procedure of Updyke et al., *J. Immunol. Meth.*, 73, 83-95 (1984).

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The heparanase-peptide was alkylated with iodoacetamide to inactivate the sulfydryl group at the C-terminus of the peptide and used to compete with anti-heparanase antibodies as a control in an immunoprecipitation experiment. The reaction was carried out at a concentration of 2 mM peptide, 1 mM dithiothreitol, 20 mM iodoacetamide, in 50 mM Tris, 150 mM sodium chloride, pH 7.5, at 25°C for 1 hr. The sample was concentrated and the alkylated peptide was separated on a Bio-Gel P-2 column (mesh 400). The void volume was collected and lyophilized.

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Cells and cell culture

Human A375-MM and A375-SM melanoma cell lines (Kozlowski et al., *J. Natl. Cancer Inst.*, 72, 913-917 (1984), which were selected in nude mice for increased

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lung colonizing potential, were a gift of Dr. I.J. Fidler (University of Texas M.D. Anderson Cancer Center, Houston, TX) Murine B16-BL6 melanoma cells, sequentially selected for increased bladder invasion (Hart, Am. J. Pathol., 97, 587-600 (1979), were from Dr. I.R. Hart (Imperial Cancer Research Laboratories, London, England). Cells were cultured in 1:1 (v/v) mixture of DME/F12 supplemented with 5% heat inactivated FBS in a tissue culture incubator gassed with 5% CO₂ at 37°C. Cells were grown in multichamber slides (Miles Scientific, Naperville, IL) for immunocytochemical studies.

Immunoprecipitation and Autoradiography

Melanoma cells were plated onto 10-cm tissue culture dishes. At subconfluency, the medium was aspirated and the cells were gently rinsed with Dulbecco's phosphate buffered saline (DPBS) once and then supplemented with DME/F12 plus 5% dialyzed FBS in the presence of 50 µCi/ml of [³⁵S]methionine (ICN, Irvine, CA). After 24 hr of metabolic labeling, the cells were rinsed with DPBS and incubated for 10 min with 2 mM EDTA in Ca²⁺, Mg²⁺-free PBS. The cells were then centrifuged at 600 x g for 5 min, the pellet lysed with 0.5% Triton X-100, 50 mM Tris-HCl, 1 mM PMSF, 5 mM NEM pH 7.5, at a ratio of 1 ml per 6 x 10⁶ cells, and the incubation continued at 25°C for 10 min with mixing on a Vortex machine. The specific activity was then determined by measuring trichloroacetic acid-precipitable radioactivity, and the protein concentration of the cell lysate was determined. The specific activity was higher than 5 x 10⁶ cpm/µg of protein. A cell lysate containing 200 µg of anti-heparanase antibodies for 1 hr at 4°C, followed by incubation with 10 µl of Streptavidin-Agarose (BRL, Bethesda, MD) for 1 hr at 4°C with mixing on a Vortex machine. The samples were washed once with 10 mM HEPES, 150 mM NaCl, pH 7.5, containing 0.5% Triton X-100,

-40-

followed by washing with 10 mM HEPES, 150 mM NaCl, pH 7.5, 4 times. The precipitates were suspended in SDS sample buffer in the presence or absence of 2.5% β -mercaptoethanol and heated at 100°C for 3 min. They were then electrophoresed on a 7.5% polyacrylamide gel (Bio-Rad, Richmond, CA) in the presence of SDS. The gel was then rinsed briefly with deionized water, dried, and placed with Kodak XOMAT film on an intensify screen for 2 days at -80°C prior to developing. 14 C-labeled molecular weight standards (Sigma) were included in the electrophoresis.

Mouse and human melanoma tissues

Mouse metastatic melanoma tissues were prepared as follows. Subconfluent B16-BL6 cell monolayers were rinsed with DPBS and incubated with 2 mM EDTA in Ca^{2+} , Mg^{2+} -free PBS for 10 min, suspended as single cells in DME/F12, chilled in an ice-water bath before centrifugation, and resuspended in cold DME/F12 at a concentration of 2.5×10^5 cells/ml. Just prior to injection, the cell suspension was warmed to room temperature, and cell aliquots (5×10^4 cells in 0.20 ml) were injected into the lateral tail vein of 6- to 8-week-old C57/BL6 mice using a 27-gauge needle. Ten mice were used in each experiment. After 2-3 weeks, the mice were exsanguinated under anesthesia and the lungs were carefully taken for frozen sectioning.

Human melanoma tissues were obtained from seven patients diagnosed with malignant melanoma. Human nevi biopsies were contributed by normal donors. Frozen tissue sections were prepared as described below.

Indirect immunocytochemistry and immunohistochemistry

Melanoma cells grown on multichamber slides (Miles Scientific) were fixed at 25°C for 20 min with 2%

paraformaldehyde in PBS, and then rinsed three times with PBS. Some cells were permeabilized with cold acetone at -20°C for 3-10 min and rinsed 3 times with PBS at 25°C. The fresh mouse and human tissues were mounted on stubs with O.C.T. mounting medium (Miles Scientific) in the vapor phase of liquid nitrogen and subsequently sectioned (2 μ m thickness) on a cryostat microtome (Damon/IEC custom microtome, Needham Heights, MA). Sections were fixed with 2% paraformaldehyde in PBS at 25°C for 20 min. After 3 rinses with PBS, the slides were incubated for 1 hr with preimmune rabbit IgG and then with PBS, anti-heparanase antibodies, or anti-heparanase antibodies pre-incubated with synthetic peptide antigens for 1 hr at 25°C. Following 3 rinses with PBS, the slides were incubated with peroxidase-conjugated goat anti-rabbit IgG (Calbiochem, La Jolla, CA) at a 1:150 dilution in PBS or PBS for 1 hr. The slides were then developed in 3-amino-9-ethylcarbazole (AEC; Sigma) buffer: 4 mg of AEC dissolved in 1.0 ml of N,N-dimethylformamide (Aldrich) in 100 ml 0.1 M sodium acetate, pH 5.2, plus of 1-2 drops 30% H₂O₂ for 20 min. The slides were covered with cover slips using glycerol-gelatin mounting medium prepared by dissolving 10 g of gelatin (Fisher Scientific) in 60 ml distilled water with heating until the gelatin dissolved, and supplementing the gelatin solution with 70 ml glycerin and 1 ml phenol.

Glycans and enzymes. Bovine lung heparan sulfate was (HS) a kind gift from Dr. N. Di Ferrante (Baylor College of Medicine, Houston, TX) and its average M_r was determined as 34,000 by sedimentation equilibrium (Nakajima, M., et al., (1984) J. Biol. Chem. V 259, pp 2283-2290 and Irimura, T., et al., (1983) Anal. Biochem. V 130, pp 461-468). Heparin (M_r 11,000) from porcine mucosal tissue was kindly donated by Drs. M. B. Mathews, J. A. Cifonelli, and L. Roden (University of Chicago,

IL). Chondroitin 6-sulfate (C6S) from shark cartilage was obtained from Miles Scientific (Naperville, IL) and further purified by gel chromatography; its average M_r was determined as 60,000 as described previously (Irimura, T., et al., (1983) Anal. Biochem. V 130, pp 461-468). Heparin from bovine lung and porcine intestinal mucosa and N-acetyl-D-glucosamine were obtained from Sigma Chemical Co. (St. Louis, MO). Monosialosyl biantennary complex-type glycopeptide UB-I-b (M_r 2190) was prepared from thyroglobulin (Sigma) (Irimura, T., et al., (1983) Anal. Biochem. V 130, pp 461-468). Heparitinase from Flavobacterium heparinum (EC4.2.2.8) was obtained from Miles Scientific.

15 High-speed gel permeation chromatography. High-speed gel permeation chromatography was carried out using a high pressure liquid chromatograph system (LDC, Riviera Beach, FL) equipped with two sequential columns (0.7 x 75 cm) of Fractogel (Toyopearl) TSK HW-55(S) (MCB, Gibbstown, NJ) as described previously (Irimura, T., et al., (1983) Anal. Biochem. V 130, pp 461-468). A one hundred microliter aliquot of sample solution was delivered into the injection port, and the chromatographic elution was performed with 0.2 M sodium chloride at a flow rate of 1.0 ml/min at 55°C (Irimura, T., et al., (1983) Anal. Biochem. V 130, pp 461-468). In the analysis of radiolabeled materials, fractions corresponding to each 36 s of elution (0.6 ml) were collected and mixed with 3.0 ml of Liquiscint (National Diagnostics, Comerville, NJ), and counted on a Beckman LS 2800 liquid scintillation counter (Beckman Instruments, Irvine, CA).

35 Cellulose acetate electrophoresis. Glycosaminoglycans were analyzed by cellulose acetate electrophoresis according to the method of Cappelletti et al.

(Cappelletti, et al., Anal. Biochem., V 99, pp 311-315).

Titan III Zip Zone cellulose acetate plates (6.0 x 7.6 cm, Helena Laboratories, Beaumont, TX) were used, and
5 electrophoresis was carried out at 70 V for 60 min in 0.5 M pyridine-acetate (pH 5.0), instead of 0.1 M barium acetate buffer employed by Cappelletti et al.
(Cappelletti, et al., Anal. Biochem., V 99, pp 311-315).
During the electrophoresis the buffer and cellulose
10 acetate plates were kept below 4°C using petroleum ether cooled with ice.

N-Desulfation and acetylation of HS. N-desulfation of HS was conducted by the methods of Nagasawa and Inoue
15 (Nagasawa et al., (1977) Methods in Carbohydr. Chem. V 8, pp 291-294). The sodium salt of purified HS was converted to the pyridinium salt by cation exchange chromatography on a column of AG50WX8(H⁺ form, Bio-Rad, Richmond, CA) and neutralization with pyridine. Complete
20 N-desulfation and partial N-desulfation of HS was carried out by solvolysis of the pyridinium salt of HS in 95% dimethylsulfoxide (DMSO) and 5% water for 120 min at 50°C, and for 60 min at 20°C, respectively. The pH of the reaction mixture was adjusted to 9.0 by the addition
25 of 0.1 M sodium hydroxide; and then the mixture was dialyzed against running tap water overnight and then against distilled water for 20 h. The N-acetylation of N-desulfated HS was performed in 4 M sodium acetate, pH 8.0, containing 4% acetic anhydride, 15% methanol for 3 h
30 at 4°C. The reaction mixture was dialyzed against running tap water overnight and then against distilled water, and the mixture was then lyophilized.

Radioisotope labeling of HS. To study the effects
35 of chemical modification of HS on its susceptibility to melanoma heparanase, HS was labeled with tritium at the

reducing end as described previously (Nakajima, M., et al., (1984) J. Biol. Chem. V 259, pp 2283-2290). One milligram of purified HS was reduced with 2 mCi of NaB[³H]₄ (340 mCi/mmol; New England Nuclear, Boston, MA) in 5 0.1 M sodium borate buffer, pH 8.0, for 5 h at 25°C. After acidification to pH 5 with acetic acid, the reaction mixture was chromatographed on a column (1.0 x 105 cm) of Sephacryl S-200 equilibrated with 0.2 M pyridine-acetate buffer, pH 5.0. Fractions of ³H-labeled 10 HS with specific M_r were collected and lyophilized. To synthesize radiolabeled HS for a solid-phase heparanase substrate, partially N-desulfated HS was N-acetylated with [1-¹⁴C]acetic anhydride. Fifteen milligrams of partially N-desulfated HS were incubated with 0.15 mCi of 15 [1-¹⁴C]acetic anhydride (10.0 mCi/mmol; New England Nuclear) in 4 M sodium acetate, pH 8.0, for 4 h at 4°C; and then further incubated with 4% acetic anhydride in the same buffer for 4 h at 4°C. The reaction mixture was extensively dialyzed against distilled water, and then 20 lyophilized. High M_r fractions of partially N-desulfated N-[¹⁴C]acetylated HS (PNDS-N[¹⁴C]Ac-HS) were obtained by gel chromatography on a column of Sephacryl S-200 as described above.

25 Reductive Amination and Coupling of ¹⁴C-labeled HS to amino-reactive agarose beads. The reducing terminal saccharides of ¹⁴C-labeled HS were reductively aminated as follows. PNDS-N[¹⁴C]Ac-HS (5 mg) was dissolved in 5 ml of distilled water and mixed with 5 ml of 4 M ammonium 30 formate and 0.8 M sodium cyanoborohydride in methanol, and then incubated at 50°C for 7 days. The reaction mixture was dialyzed against distilled water and lyophilized. The reductively aminated products were dissolved in 10 ml of 0.1 N sodium bicarbonate, pH 8.5, 35 and mixed with Affi-Gel 15 (or Affi-Gel 10; Bio-Rad) prepared from the original suspension by successive

washing in 2-propanol and then ice-cold distilled water. The mixture was incubated at 4°C for 24 h with gentle mixing. The pH was then adjusted to pH 8.5 with 0.1 N sodium bicarbonate and the incubation further continued.

- 5 After 24 h the unreacted sites on the Affi-Gel 15 were blocked by addition of 1 ml of 1 M glycine ethyl ester (pH 8.0), and the beads were again incubated for 6 h at 4°C. After the reaction was complete, the coupling products were extensively washed in 1.5 M sodium
- 10 chloride, and incubated in 0.1 M sodium acetate, 0.15 M sodium chloride, 0.2% Triton X-100, and 0.05% sodium azide (pH 6.0) at 37°C overnight. The products were further washed in the same buffer and stored at 4°C.

- 15 The summary of the above described synthetic procedures is shown in Figure 1.

Chemical deacetylation and radioactive reacetylation of heparan sulfate and its coupling to agarose beads.

- 20 ¹⁴C-or ³H-labeled HS were prepared by chemical deacetylation and radioactive reacetylation as follows. Nine milligrams of bovine lung HS (provided by Dr. N. Di Ferrante, Baylor College of Medicine, Houston, Tx.) were dried with 1 mg hydrazine sulfate over phosphorous
- 25 pentoxide under vacuum at 50°C for 48 hrs. Anhydrous hydrazine (0.5 mg, Pierce Chemical, Rockford, Il.) was added to the dried HS, and the mixture was heated in a tightly screwed tube under nitrogen atmosphere at 100°C for 1 hr. After the reaction, the hydrazine was removed
- 30 by repeated evaporation with toluene over sulfuric acid dessicant under vacuum conditions. To separate deacetylated HS from residual reagents and partial degradation products, completely dried residue was dissolved in 0.5 ml water and subjected to gel filtration on a 0.8 x 30 cm
- 35 column of BioGel p-10 (400 mesh) eluting with distilled water. The void volume fraction was collected and

lyophilized. The yield was approximately 60% by weight. The N-deacetylated HS was then N-acetylated with 50 uCi [¹⁴C]-acetic anhydride (10 mCi/mmol: NEN, Boston, Ma.) or 5 mCi ³H-acetic anhydride (400 mCi/mmol:NEN) in 0.5 ml of 4 M sodium acetate for 18 hrs. Complete N-Acetylation was accomplished by mixing with 0.1 ml of non-labeled acetic anhydride for 1 hr. ¹⁴C- or ³H-labeled HS was purified on the same BioGel P-10 column as described above.

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For the solid-phase heparanase assay, ³H-HS was aminated at the reducing terminal with 2 M ammonium acetate in the presence of 0.4 M sodium cyanoborohydride in 50% methanol at 50°C for 6 days. Aminated ³H-HS was purified by gel filtration as above, and the resulting solution was made to 0.1 M in sodium carbonate. To 10⁶ cpm of aminated ³H-HS, 1.0 ml Affi-Gel 15 was added after the gel beads were washed with isopropanol and chilled water according to the manufacturer's recommendations. The coupling reaction was continued at 4°C for 48 hrs with continuous agitation. The agarose beads were then washed with 4 M sodium chloride repeatedly to remove non-covalently attached ³H-HS from the beads.

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Melanoma cells and cell culture. The high lung-colonizing metastatic murine B16 melanoma subline (B16-F10) and fourteen established cell lines of human malignant melanoma were employed in this study. The human melanoma cell lines used were: SK-MEL-19, SK-MEL-23, SK-MEL-93(DX1), SK-MEL-93(DX3), SK-MEL-93(DX6), Hs294T, Hs852T, HS939, T294, M40, RON, BMCL, A375 parent, and A375 Met Mix. A375 Met Mix cells were prepared from spontaneous lung metastases of A375 parental cells in the athymic nude mice and both A375 cell lines were provided by Dr. I.J. Fidler (The University of Texas-M.D. Anderson Hospital and Tumor Institute, Houston, TX). Melanoma

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cells were grown on plastic tissue culture dishes in a 1:1 mixture of Dulbecco's modified minimum essential medium and Ham's F12 medium (DMEM/F12; Gibco Laboratories, Grand Island, NY) with 10% fetal bovine serum (Hyclone, Sterile Systems, Inc., Logan, UT) and without antibiotics, under humidified conditions in a 95% air-5% CO₂ atmosphere. All cell cultures were determined to be free of mycoplasma contamination with the use of mycoplasma detection system (BRL MycoTest; Bethesda Research Laboratories, Gaithersburg, MD).

Preparation of cell extracts. Subconfluent melanoma cells were harvested by treatment for 10 min with 2 mM ethylene diamine tetracetic acid (EDTA) in Ca²⁺, Mg²⁺-free DPBS. Single cell suspensions were washed twice by brief centrifugation in 0.14 M sodium chloride, 10 mM Tris-HCl buffer, pH 7.5, and checked for viability (usually >95%) by trypan blue dye exclusion. Cells were suspended in chilled 50 mM Tris-HCl buffer, pH 7.5, containing 0.5% Triton X-100 at a concentration of 6 x 10⁶ cells/ml and extracted for 5 ml at 25°C and for an additional 1 h at 4°C. The supernatant was collected after centrifugation at 9800 x g for 5 min at 4°C. Protein contents in the centrifugated extracts were determined by a modification of the Lowry technique to correct for the presence of Triton X-100 in the samples (Nakajima, M., et al., (1984) J. Biol. Chem. V 259, pp 2283-2290).

Enzymatic degradation of unmodified and modified HS.
In the enzymatic degradation experiments the ³H-labeled HS substrate (10 ug) was incubated with a B16-F10 cell extract (80 ug protein) in 200 ul of 0.1 M sodium acetate buffer (pH 6.0) containing 0.15 M sodium chloride, 0.2% Triton X-100 and 0.05% sodium azide (reaction buffer A) in the presence of 20 mM D-saccharic acid 1,4-lactone (SAL, a potent exo-β-glucuronidase inhibitor). The

incubation was carried out at 37°C with gentle mixing, and was terminated by chilling the mixture to 4°C and adding 20 ul of trichloroacetic acid to a final concentration of 5%. The supernatant was obtained by
5 centrifugation at 9800 x g for 5 min and it was subjected to analysis by high-speed gel permeation chromatography.

Heparanase assay using a solid-phase substrate. A suspension of PNDS-N[¹⁴C]Ac-HS coupled to Affi-Gel 15 was
10 mixed with a melanoma cell extract and incubated in 400 ul of reaction buffer A containing 20 mM SAL. The enzyme reaction was terminated by chilling the solution to 4°C and mixing it with 40 ul of 50% trichloroacetic acid. After incubation for 10 min at 4°C, the mixture was
15 centrifuged at 9800 x g for 5 min, and the supernatant was withdrawn. Two hundred microliter aliquots of the supernatant were mixed with 55 ul of 1.0 N sodium hydroxide and 4 ml of Liquiscint (National Diagnostic) and counted in a Beckman LS 2800 liquid scintillation
20 counter.

Effects of N-desulfation and N-acetylation of HS on HS degradation by melanoma heparanase. To label purified
25 HS with radioactive molecules without use of linking reagents that might cause significant changes in HS molecular structure, we replaced some of the N-sulfate groups with N-[¹⁴C]acetyl groups. This idea was based on our previous observation that B16 melanoma heparanase was highly active against various HS molecules but inactive
30 against heparin, and that HS differs from heparin in its high N-acetyl and low N-sulfate contents.

The effect of N-desulfation and N-acetylation of HS on its susceptibility to melanoma heparanase was assessed
35 using HS labeled with tritium at the reducing terminal saccharide. Since HS purified from bovine lung had

mostly *N*-acetyl-D-glucosamine at the reducing ends (Nakajima, M., et al., (1984) J. Biol. Chem. V 259, pp 2283-2290), HS was reduced with NaB[³H],. HS labeled with tritium at the reducing end was *N*-desulfated by

5 solvolysis with DMSO, and *N*-desulfated [³H]HS was then *N*-acetylated with acetic anhydride. These three ³H-labeled, chemically modified HS molecules were analyzed by cellulose acetate electrophoresis in 0.5 M pyridine-acetate buffer, pH 5.0. The relative mobilities of HS *N*-desulfated HS (NDS-HS) and *N*-desulfated *N*-acetylated HS (NDS-NAC-HS) under the electrophoresis conditions

10 described in the materials and methods were 3.30, 2.55, and 2.90, respectively. These findings indicated that *N*-desulfation of HS resulted in a significant loss of negative charge; however, the total negative charge was partially recovered by acetylation of free amino groups. The average molecular size of NDS-HS and NDS-NAC-HS were determined by high-speed gel permeation chromatography, and were found to be 30,000 and 31,500, respectively

20 (Fig. 2). Each of HS, NDS-HS and NDS and NDS-NAC-HS was incubated with B16 melanoma cell extracts in the presence of SAL (a potent α -glucuronidase inhibitor), and the incubation products were analyzed by high-speed gel permeation chromatography. All these substrates were

25 cleaved by melanoma heparanase at high rates and the elution profiles of their degradation products were similar, although the *M_r* of the fragments produced were characteristic for each substrate (Fig. 2). Degradation of each of the chemically modified HS was totally

30 inhibited by addition of amounts of heparin purified from bovine lung or porcine intestinal mucosa (data not shown). The results indicated that *N*-sulfate in HS may not be important for its recognition and cleavage by melanoma heparanase, and that the chemical modification

35 of sulfated amino groups in HS does not significantly affect its degradation by heparanase.

N-Desulfated and N-acetylated heparin. The known structures of HS and heparin suggested that N-desulfation and subsequent N-acetylation of heparin may generate local structures similar to those present in HS. Heparin is a potent inhibitor of B16 melanoma heparanase (Nakajima, M., et al., (1984) J. Biol. Chem. V 259, pp 2283-2290); however, its heparanase inhibitory activity is lost by the removal of N-sulfate (Irimura, et al. (1985) J. Cell. Biochem. V 9A, p 148). Since the results above suggested that N-sulfate in HS is unnecessary for its cleavage by melanoma heparanase, N-desulfated N-acetylated heparin was used as a heparanase substrate. Heparin from porcine intestinal mucosa (M_r 11,000) previously labeled with tritium at its reducing end (Nakajima, M., et al., (1984) J. Biol. Chem. V 259, pp 2283-2290) was N-desulfated and then N-acetylated by the procedures employed in the preparation of NDS-NAC-HS. The product, N-desulfated N-acetylated heparin (NDS-NAC-heparin), had an apparent M_r of about 10,500 as determined by high-speed gel permeation chromatography; and its relative electrophoretic mobility on cellulose acetate in 0.2 M pyridine-acetate buffer, pH 5.0, was 0.87 when the electrophoretic mobility of ^3H -labeled heparin was taken as 1.00. ^3H -labeled heparin and ^3H -labeled NDS-NAC-heparin were incubated with B16 cell extracts and the reaction products were analyzed by high-speed gel permeation chromatography. Percentage degradation of the original substrates was calculated from the decrease in area of the high M_r half of the substrate peak as reported previously (Nakajima, M., et al., (1984) J. Biol. Chem. V 259, pp 2283-2290). During the first 6 h incubation with a B16 cell extract, less than 5% of heparin was degraded, while approximately 20% of the NDS-NAC-heparin was fragmented. However, NDS-NAC-heparin was not further cleaved and the major peak of NDS-NAC-heparin on high-

speed gel permeation chromatography did not shift to a lower M_r even after a prolonged incubation. This suggested that N-desulfation and subsequent N-acetylation of heparin can result in the generation and/or exposure of heparanase-susceptible glucuronosyl linkages in a part of the heparin molecule. Thus, NDS-NAC-heparin cannot be utilized as a melanoma heparanase assay substrate.

Synthesis of solid-phase substrate for melanoma heparanase assay. The procedure for the synthesis of a solid-phase substrate for heparanase is illustrated in Fig. 1. To minimize the radiolabeling effects on the HS structure, HS was only partially N-desulfated by solvolysis in 95% DMSO and 5% H₂O for 1 h at 20°C. Partially N-desulfated HS was acetylated with [1-¹⁴C]acetic anhydride as described in the material and methods section. The remaining free amino groups were completely acetylated with acetic anhydride. These steps yielded PNDS-N[¹⁴C]Ac-HS, (M_r 33,000) with radioactivity of 294 cpm/ug. The relative mobility of PNDS-N[¹⁴C]Ac-HS on cellulose acetate electrophoresis was 3.15, indicating that the total negative charge of PNDS-N[¹⁴C]Ac-HS is much closer to that of unmodified HS than is that of NDS-NAC-HS as expected.

The reducing terminal saccharides of PNDS-N[¹⁴C]Ac-HS were reductively aminated with 2M ammonium formate and 0.4 M sodium cyanoborohydride in 50% methanol. The products having free amino groups only on the reducing termini were then coupled to aminoreactive agarose beads such as Affi-Gel 10 or Affi-Gel 15. Incubation of 5 mg of PNDS-N[¹⁴C]Ac-HS with 0.5 ml of packed Affi-Gel 15 resulted in the immobilization of 10.2% PNDS-N[¹⁴C]Ac-HS onto Affi-Gel 15 (0.51 mg PNDS-N[¹⁴C]Ac-HS per 0.5 ml of gel). Increasing concentrations of PNDS-N[¹⁴C]Ac-HS up to 10 mg per 0.5 ml of Affi-Gel 15 did not significantly

affect the coupling efficiency under the conditions used.

PNDS-N[¹⁴C]Ac-HS was also conjugated to Affi-Gel 10 under the same conditions used for the coupling of PNDS-N[¹⁴C]Ac-HS to Affi-Gel 15. However, the coupling efficiency was low, less than 1%, between pH 7.5 and 8.5. Therefore, a positive charge spacer at the aminoreactive site of Affi-Gel 15 may be important in the effective coupling of PNDS-N[¹⁴C]Ac-HS to Affi-Gels. Using Affi-Gel 15, one of the best heparanase assay substrates was produced: PNDS-N[¹⁴C]Ac-HS immobilized on agarose through only one covalent linkage at the reducing terminal end.

Enzymatic degradation of PNDS-N[¹⁴C]Ac-HS immobilized on agarose gel beads. The susceptibility of PNDS-N[¹⁴C]Ac-HS immobilized on agarose gel to HS degrading enzymes was examined by incubating the substrates (4500 cpm, 15 ug) with bacterial heparitinase (EC.4.2.2.8) at a concentration of 5 units/ml in 0.1 M sodium acetate buffer, pH 7.0, containing 1 mM calcium acetate (Linker, et al. (1972) Methods Enzymol. V 28, pp 902-911). Most of ¹⁴C activity (82%) appeared in the supernatant of the incubation mixture after a 24 h incubation, indicating that PNDS-N[¹⁴C]Ac-HS immobilized on agarose is very susceptible to HS degrading enzymes. The remainder of the PNDS-N[¹⁴C]Ac-HS was not released from the gel, even after prolonged incubation. This could be explained by the limitation of using a Flavobacterium heparitinase. The same amount of substrate (4500 cpm) was incubated with B16 cell extract for various periods in the presence of 20 mM SAL to prevent the sequential degradation by exoglycosidases. The relationships between the amounts of cell extract (ug protein) added and the release ¹⁴C activity are shown in Fig. 3. In this case, the maximum amount of released ¹⁴C activity were 56% of the total ¹⁴C activity present in the solid-phase substrates. A

portion of the ^{14}C activity could not be released by melanoma heparanase, since the incubation of HS or of chemically modified HS with a B16 cell extract resulted in the production of large fragments with the original reducing termini (Fig. 2). A linear relationship between the amount of cell extract added and the release ^{14}C activity was found for each incubation period (Fig. 3). Since the results from the 12 h incubation were linear over the widest range of cell extract amounts, we measured the degradation of PNDS-n[^{14}C]Ac-HS during a 12 h incubation.

The effect of heparin on the degradation of the solid-phase substrates was investigated by addition of substrate-equal amount (15 ug) of heparin from porcine intestinal mucosa or heparin from bovine lung to the incubation mixture containing B16-F10 cell extract (80 ug protein) and PNDS-N[^{14}C]Ac-HS immobilized on agarose. The addition of either heparin caused up to 80% inhibition of the degradation of the solid-phase substrates, consistent with our previous results (Nakajima, M., et al., (1984) J. Biol. Chem. V 259, pp 2283-2290).

Measurement of heparanase activity in human melanoma cells by use of PNDS-N[^{14}C]Ac-HS immobilized on agarose beads. Using PNDS-N[^{14}C]Ac-H immobilized on agarose beads, the following fifteen human melanoma cell lines were tested for heparanase activity: SK-MEL-19, SK-MEL-23, SK-MEL-93(DX1), SK-MEL-93(DX3), SK-MEL-93(DX6), Hs24T, Hs852T, Hs939, T294, M40, RON, BMCL, A375 parent, A375 Met Mix, and A375 M6. All the human melanoma cells showed the ability to degrade HS in the presence of SAL as shown in Table 1. Six of these human malignant melanoma cell lines such as SK-MEL-93(DX1), SK-MEL-93(DX6), Hs939, M40, A375 Met Mix, and A375 M6 demonstrated significantly greater ability to degrade HS

than did mouse B16 melanoma subline F10.

5 Interestingly, A375 Met Mix and A375 M6 cells were
selected from A375 parental cells by their ability to
colonize the lung in athymic nude mice. They were
reported to have a high metastatic potential, while A375
parental cells had a very low metastatic potential
(Kozlowski, et al. (1984) J. Natl. Cancer Inst. V 72, pp
913-917). Therefore, the heparanase activity of A375
10 cells may correlate with their spontaneous lung
metastatic potential.

The present inventors have previously found that
intact B16 melanoma cells or B16 cell extracts from
15 sublines of high lung colonization potential degrade
purified HS at higher rates than B16 cells of poor lung
colonization potential (Nakajima, et al. (1983) Science V
220, pp 611-613), and that B16 melanoma HS degrading
endoglycosidase is an endo- β -glucuronidase (heparanase)
20 (Nakajima, M., et al., (1984) J. Biol. Chem. V 259, pp
2283-2290).

TABLE 1

HEPARAN SULFATE DEGRADATION ACTIVITY
IN HUMAN MALIGNANT MELANOMA CELLS

5

	Melanoma cell lines	Heparan sulfate degradation activity* mean \pm S.D. (cpm)
10	SK-MEL-19	379 \pm 40
	SK-MEL-23	397 \pm 29
	SK-MEL-93 (DX1)	625 \pm 36
	SK-MEL-93 (DX3)	381 \pm 25
	SK-MEL-93 (DX6)	703 \pm 19
15	Hs294T	381 \pm 44
	Hs852T	202 \pm 16
	Hs939	619 \pm 44
	T294	366 \pm 15
	M40	787 \pm 75
20	RON	457 \pm 27
	BMCL	118 \pm 31
	A375 parent ^b	392 \pm 38
	A375 Met Mix ^b	659 \pm 22
	A375 M6 ^b	612 \pm 48
25	B16-F10 (mouse melanoma)	510 \pm 34

*Heparanase assay was carried out by the incubation of a Triton X-100 cell extract (2.4×10^5 cells) with PNDS-N[¹⁴C]Ac-HS immobilized on agarose beads (4500 cpm) at 37°C for 12 h. The details of experiment are described in the materials and methods section. The radioactivity released in the presence of heat inactivated enzymes was subtracted from the raw data.

35

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^bA375 Met Mix and A375 M6 cells derived from lung metastases of A375 parental cells in athymic nude mice possess highly spontaneous lung metastatic potential, while A375 parental cells have very low spontaneous metastatic potential.

Heparanase Activity in Sera from Tumor Bearing Hosts

Preparation of blood sera. Blood was withdrawn by venepuncture without anticoagulant and allowed to clot for 1 hr at 22°C. Samples were centrifuged at 4°C for 10 min at 800 x g and for 15 min at 1600 x g. The resultant sera were divided into small aliquots and snap-frozen in liquid nitrogen, and then maintained at -80°C until analyzed.

Assay of sera for heparanase. The serum was diluted 5-fold with 0.1 M sodium acetate buffer, pH 6.0, containing 0.15M sodium chloride. Two hundred microliter aliquot of the diluted serum was mixed with 200 ul of radiolabeled solid phase substrate suspension (3,000 cpm, 10 ug) in the same buffer and incubated at 37°C. At various incubation periods the enzyme reaction was terminated by chilling to 4°C, and the reaction sample was mixed with 40 ul of 50% trichloroacetic acid. The mixture was incubated for 10 min at 4°C and centrifuged at 9800 x g for 5 min. A two hundred microliter aliquot of the supernatant was withdrawn, neutralized with 55 ul of 1.0 N sodium hydroxide, and then mixed with 4 ml of Liquiscint (National Diagnostic). The radioactivity was measured by a Beckman LS 2800 liquid scintillation counter. There was a linear relationship between incubation time and enzyme reaction. The activity was reported as units per milliliter serum. One unit of activity

refers to the amounts of enzyme that liberates 1 ug of HS per minute.

5 Heparanase activity in the sera from malignant
6 melanoma patients. The sera from 20 melanoma patients
7 at the various stages of the disease and from 15 normal
8 adults were assayed for heparanase, and the results are
9 shown in Fig. 4. The mean value and standard deviation
10 of heparanase activity in the sera from melanoma
11 patients and normal adults were 0.0177 ± 0.0075 and
12 0.0096 ± 0.0025 U/ml, respectively. The sera from some
13 patients who have been treated by chemotherapy showed
14 the normal levels of heparanase activity.

15 Heparanase activity in the sera from rats bearing
16 13762NF mammary adenocarcinoma. Highly metastatic
17 mammary adenocarcinoma MTLn3 cells (1×10^6) were
18 injected subcutaneously into the left inguinal mammary
19 fat pad of age matched female F344 rats. Rats were
20 sacrificed at various periods post-injection, and the
21 size of primary tumors, the number of lung metastases,
22 and the serum heparanase activity were measured. The
23 heparan sulfate degradative activities in sera
24 increased with time after the subcutaneous inoculation
25 of MTLn3 cells (Fig. 5). The activities in sera
26 correlated with the sizes of the primary tumors
27 (correlation coefficient $r = 0.770$, Fig. 6). The sera
28 from rats with large numbers of metastases in the lymph
29 nodes and lungs demonstrated much higher heparanase
30 activities than the sera from rats with few or no meta-
31 stasis (Fig. 7).

32 Purification of melanoma heparanase. Melanoma
33 cells (murine B16 melanoma subline B16-BL6 or human
34 melanoma Hs 939 cells) were grown in a 1:1 mixture of
35 DME/F12 medium supplemented with 5% heat-inactivated

fetal bovine serum. Subconfluent cells were harvested by a treatment for 10 min with 2 mM EDTA in PBS and then washed twice in 0.14 M NaCl, 10 mM Tris-HCl buffer, pH 7.2. The following steps were performed at 4°C. Cells (2×10^8) were extracted in 30 ml of 50 mM Tris-HCl buffer, pH 7.2, containing 0.2% Triton X-100, 10 μ M PMSF (buffer A) for 1 hr. The supernatant (approximately 1.5 mg protein/ml) was collected after centrifugation at 30,000 x g for 30 min, and was loaded on a column of concanavalin A-Sepharose 4B (2 x 10 cm) equilibrated with buffer A. After washing with 10 ml of buffer A, the absorbed material was eluted with 1 M alpha-methyl-D-mannoside in buffer A. The eluents were filtered through a heparin-sepharose CL-6B column (2 x 10 cm) equilibrated with 50 mM Tris HCl buffer, pH 7.2, containing 0.15 M sodium chloride 0.2% Triton X-100. The column was washed with 100 ml of the same buffer and 100 ml of 0.15 M sodium chloride 50 mM Tris-HCl, pH 7.2, and then heparin-bound proteins were eluted with a linear salt gradient (0.15 M-1.2 M sodium chloride). The heparanase active fractions were collected and dialyzed against 0.15 M sodium chloride and 0.01 M potassium phosphate, pH 6.0. After centrifugation at 50,000 x g for 30 min, the supernatant was loaded on a hydroxylapatite column (1.5 x 45 cm) equilibrated with 0.15 M sodium chloride and 0.01 M potassium phosphate, pH 6.0. Heparanase was eluted with a linear gradient of potassium phosphate (0.01 M to 0.6 M) in 0.15 M sodium chloride, pH 6.0. The heparanase fractions were concentrated by ultrafiltration using YM-10 membranes, and were subjected to the further purification by Sepharose CL-6B gel filtration. The Sepharose CL-6B chromatography was performed in 0.15 M sodium chloride, and 20 mM potassium phosphate, pH 6.0. Further chromatography, now with Sephadex G-150, was carried out in 0.5 M sodium chloride and 25 mM Tris-HCl, pH

7.5. A single heparanase peak obtained from the Sephadex chromatography contained a glycoprotein of M_r 96,000.

5 Properties of human melanoma heparanase. Melanoma heparanase is active between pH 5.5 and 7.5 and degrades heparan sulfate but not other glycosaminoglycans. Heparin and dextran sulfate are potent inhibitors of melanoma heparanase.

10

TABLE 2

HEPARANASE SUSCEPTIBILITY AND HEPARANASE
INHIBITORY ACTIVITY OF CHEMICALLY
MODIFIED HEPARAN SULFATE AND HEPARIN

5

10	HS Glycosaminoglycans Degradation ²	Inhibition of	
		Degradation ¹	
15	Heparan sulfate (HS)	+	-
	N,Q-Desulfated HS	+	-
	N,Q-Desulfated and N-acetylated HS	+	-
	N-Desulfated HS	+	-
	N-Desulfated and N-acetylated HS	+	-
20	Heparin	-	++
	N,Q-Desulfated heparin	-	-
	N,Q-Desulfated and N-acetylated heparin		±
	N-Desulfated heparin	-	-
25	N-Desulfated and N-acetylated heparin	±	+
	N-Desulfated and N-methylated heparin	-	-
	Carboxyl reduced heparin	-	±

30

¹ ³H-labeled glycosaminoglycan was incubated with a cell extract (80 ug of protein) in 0.1 M sodium acetate buffer (pH 6.0) containing 0.15 M NaCl, 0.2% Triton X-100 and 0.05% NaN₃ for 6 hr at 37°C in the presence of 20 mM D-saccharic acid 1,4-lactone (SAL) and was then subjected to high-speed gel-permeation chromatography.

35

Percent of degradation was determined by measuring the decrease in area of the high M_r half of the glycosaminoglycan peak (see Figure 2). +, more than 80%; \pm , 5% to 15%; -, less than 5% (S.D. < 5.0%).

5

2 Five micrograms of unlabeled glycosaminoglycan was added to the incubated mixture of 3H -labeled HS from bovine lung and a cell extract. Inhibition of HS degradation was determined by measuring the decrease in area of the high M_r half of the HS peak. ++, more than 80% inhibition; +, 25-50% inhibition; \pm , 5-25% inhibition; -, less than 5% inhibition (S.D. < 5%).

10

Human and Murine Heparanase Are Antigenically Related

15

Anti-heparanase antibodies directed against the amino terminus of murine melanoma heparanase were prepared as described above and some of the antibodies were subsequently biotinylated. In an immunoprecipitation experiment, human A375-SM (Fig. 9a-d) and mouse B16-BL6 (Fig. 9e-h) melanoma cell lysates metabolically labeled with [^{35}S]methionine were first incubated with biotinylated anti-heparanase antibodies (Fig. 9b,c,f,g) and then with streptavidin-agarose. The antibody specifically immunoprecipitated a major

20

25

30

The specificity of anti-heparanase antibodies is demonstrated in Fig. 9. The M_r -97,000 bands were not noted in the presence of excess amount of competing heparanase N-terminal peptide (Fig. 9a,e). Proteins of

35

related to streptavidin-agarose contaminants (Fig. 9d,h). In addition to these non-specific protein bands, a faint band of M_r -105,000 (Fig. 9b,c,e,f) appeared to be related to anti-heparanase antibody precipitation but its precipitation could not be inhibited by excess amount of heparanase peptide ((Fig. 9a,e). Therefore, it was considered to be a nonspecific component.

The antibodies immunoprecipitated a human M_r -97,000 protein from A375SM cell lysates just as well as that from B16-BL6 mouse melanoma cell lysates. Therefore, the human and mouse heparanase molecules are similar in molecular weight and antigenically related. To further confirm the specificities of the antibodies against heparanase, we examined the immunoprecipitates on 2-dimensional gels. The pI of the immunoprecipitates were the same as that of the purified heparanase: 5.0-5.2.

Heparanase Molecules are Immunochemically Localized at the Melanoma Cell Surface and in the Cytoplasm

Acetone-permeabilized human A375-SM (Fig. 10a) and mouse B16-BL6 melanoma cells were intensely stained with the anti-heparanase antibodies in an indirect immunochemical assay. The immunocytochemical staining of A375-SM and B16-BL6 cells with anti-heparanase antibodies appeared to be heterogeneous from cell to cell (Fig. 10a, b). Nonpermeabilized A375SM (Fig. 10b) and B16-BL6 cells, in which the antibodies can only interact with antigens on the cell surface, were also stained although not as intensely as their permeabilized counterparts. The B16-BL6 cells were stained essentially the same as the A375SM cells; however, the

presence of melanin deposits sometimes interfered with the immunoprecipitates. In contrast to the intense staining of the melanoma cells by anti-heparanase antibodies, there was no detectable immunoprecipitates.

5 In contrast to the intense staining of the melanoma cells by anti-heparanase antibodies, there was no detectable immunocytochemical staining in any of the controls (Fig. 10c-f), including one where anti-heparanase antibodies were preincubated with the N-

10 terminal heparanase peptide (Fig. 10d).

Mouse Melanoma Cells are Stained with Anti-heparanase Antibodies in Experimental Metastatic Tumors

Using immunohistochemical techniques, heparanase was

15 localized in experimental mouse metastatic melanoma tumors. Frozen sections of mouse lung tissue containing micrometastatic melanoma nodules were specifically stained with anti-heparanase antibodies. Examination showed heparanase antigen localized in the tumor, not in

20 normal tissue (Fig. 11f). The sections presented in Fig. 11d-f were taken from different levels of the same micrometastatic tumor nodule. Serial sections show the morphology of the micrometastatic lesion in the upper right corner of the micrograph (Fig. 11d). The

25 micrometastatic melanoma nodule appeared to contain large quantities of melanin, and the melanoma cells appeared to be heterogeneous in size and have larger

nuclei than the adjacent pulmonary alveolar epithelial cells. The micrometastatic melanoma nodules were intensely stained with anti-heparanase antibodies, and the intensity of staining in the metastatic melanoma tissue was higher than that in the adjacent lung tissue (Fig. 11f). The same set of controls as those shown in Fig. 2 was carried out, but only a representative control is presented (Fig. 11e). No staining was found in any of the controls, and the presence of melanin in melanoma nodules was profound.

Metastatic and Invasive Melanoma Cells are Stained with Anti-heparanase Antibodies in Human Metastatic Melanoma Tissues

Human melanoma tissues from seven patients with metastatic melanomas were examined for the presence of heparanase using immunohistochemical techniques. The melanoma cells were stained with anti-heparanase antibodies, and in each case the surrounding tissues, including connective tissue, were essentially unstained. As a representative case, a cutaneous melanoma is presented (Fig. 11a-c). The malignant melanoma cells, which had invaded dermis connective tissue, are shown adjacent to an arteriole and remnants of collagen fibers (Fig. 11a). In this case, the tumor cells were amelanotic and heterogeneous in size. Anti-heparanase antibodies that stained melanoma cells did not stain

surrounding normal parenchymal cells except slightly the endothelial cells in a blood vessel (Fig. 11c). The same controls as those shown in Fig. 10 were performed, but only a representative one is presented (Fig. 11b).

5 In addition, human nevi were examined for the presence of heparanase antigen using the same procedure as the melanoma tissues. The melanocytes in the nevi and adjacent normal skin were not stained by the anti-heparanase antibodies.

10

Changes may be made in the construction, operation and arrangement of the various parts, elements, steps and procedures described herein without departing from the concept and scope of the invention as defined in the
15 following claims.

CLAIMS:

1. An immunoassay method for detecting the presence of
a glycosaminoglycan endoglycosidase comprising
5 combining a human biological sample suspected of
containing glycosaminoglycan endoglycosidase with at
least one antibody specifically binding the
glycosaminoglycan endoglycosidase and determining
binding of said antibody to the sample as indicative of
10 glycosaminoglycan endoglycosidase amount and
localization.
2. The method of claim 1 wherein the antibody is
15 polyclonal.
3. The method of claim 1 wherein the antibody is
directed toward an N-terminal region of the
20 glycosaminoglycan endoglycosidase.
4. The method of claim 1 wherein the immunoassay is a
radioimmunoassay, an enzyme immunoassay, or a
25 fluorescent immunoassay.

5. The method of claim 1 wherein the glycosaminoglycan endoglycosidase is heparanase.

5 6. An immunoassay method for detecting the presence of
a heparan sulfate endoglycosidase comprising combining
a buffered aqueous solution of a human biological
sample suspected of containing heparan sulfate
endoglycosidase with at least one antibody raised to
10 the heparan sulfate endoglycosidase and determining the
level of binding of said antibody or antibodies to the
sample as indicative of the presence of heparan sulfate
endoglycosidase.

15

7. The method of claim 6 wherein the antibody is polyclonal.

20

8. The method of claim 6 wherein the heparan sulfate endoglycosidase is heparanase.

25

9. The method of claim 6 wherein the immunoassay is a radioimmunoassay, an enzyme immunoassay, or a fluorescent immunoassay.

10. A kit useful for the detection of a glycosaminoglycan endoglycosidase in a sample which comprises:

5 a carrier being compartmentalized to receive one or more container means in close confinement therein;

a first container means comprising an antibody
10 which is specific for glycosaminoglycan endoglycosidase;

a second container means comprising a detectably
15 labeled antibody which specifically binds glycosaminoglycan endoglycosidase.

11. A kit useful for the detection of glycosaminoglycan endoglycosidase in a sample which comprises:

20 a carrier being compartmentalized to receive one or more container means in close confinement therein;

25 a first container means comprising an antibody which specifically binds glycosaminoglycan endoglycosidase;

a second container means comprising detectably
labeled glycosaminoglycan endoglycosidase.

5

12. The kit of claim 10 or 11 wherein said antibody in
said first container means is immobilized on said
container means.

10

13. The kit of claim 10 wherein said antibody in said
second container is labeled with a radiolabel, an
enzyme label, biotin, a fluorescent label or a
chromophore.

15

14. The kit of claim 10 wherein the antibody in said
first container means or second container means is a
polyclonal antibody.

20

15. The kit of claim 10 or 11 which also comprises a
multiplicity of container means with different amounts
of glycosaminoglycan endoglycosidase antigen.

25

16. The kit of claim 10 or 11 wherein said first container means or second container means is a tube.

5 17. The kit of claim 10 or 11 wherein the glycosaminoglycan endoglycosidase is heparanase.

18. A kit useful for the detection of a heparan sulfate
10 endoglycosidase in a sample which comprises:

a carrier being compartmentalized to receive
one or more container means in close
confinement therein;

15 a first container means comprising an antibody
which specifically binds heparan sulfate
endoglycosidase;

20 a second container means comprising a detectably
labeled antibody specifically binding heparan
sulfate endoglycosidase.

25 19. A kit useful for the detection of heparan sulfate
endoglycosidase in a sample which comprises:

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a carrier being compartmentalized to receive one or more container means in close confinement therein;

5 a first container means comprising an antibody which specifically binds heparan sulfate endoglycosidase;

10 a second container means comprising detectably labeled heparan sulfate endoglycosidase antigen.

20. The kit of claim 18 or 19 wherein said antibody in said first container means is immobilized on said container means.

21. The kit of claim 18 wherein said antibody in said second container is labeled with a radiolabel, an enzyme label, a fluorescent label or a chromophore.

22. The kit of claim 18 wherein said antibody in said second container means is a monoclonal antibody.

25

23. The kit of claim 18 or 19 wherein the heparan

sulfate endoglycosidase is heparanase.

24. The kit of claim 18 or 19 wherein the antibody in
5 the first container means is polyclonal.

25. The kit of claim 18 or 19 which also comprises a
multiplicity of container means with different amounts
10 of heparan sulfate endoglycosidase antigen.

26. The kit of claim 18 or 19 wherein said container
means is a tube.
15

27. A peptide for immunogenically raising antibodies to
heparanase, the peptide consisting essentially of
EEDLGKSREGSRTDD.
20

28. The peptide of claim 27 defined further as being
EEDLGKSREGSRTDDC.

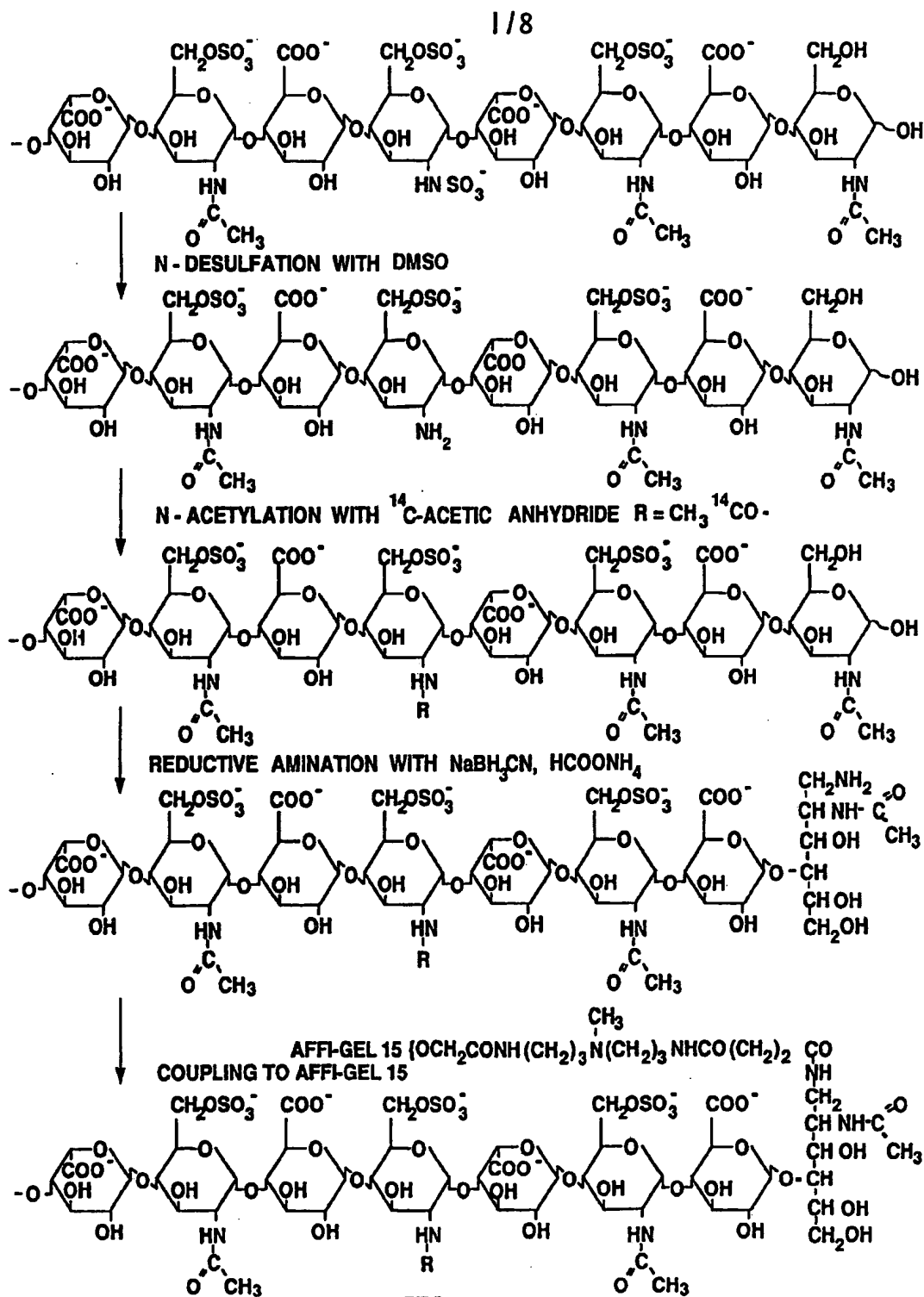
29. A method of raising antibodies specifically binding
heparanase, the method comprising immunizing an animal
25

with a carrier coupled to a peptide from the N-terminal region of heparanase.

5 30. The method of claim 29 wherein the peptide is that of claim 27 or 28.

10 31. The method of claim 29 wherein the carrier is a protein.

15 32. The method of claim 29 wherein the carrier is keyhole limpet hemocyanin.



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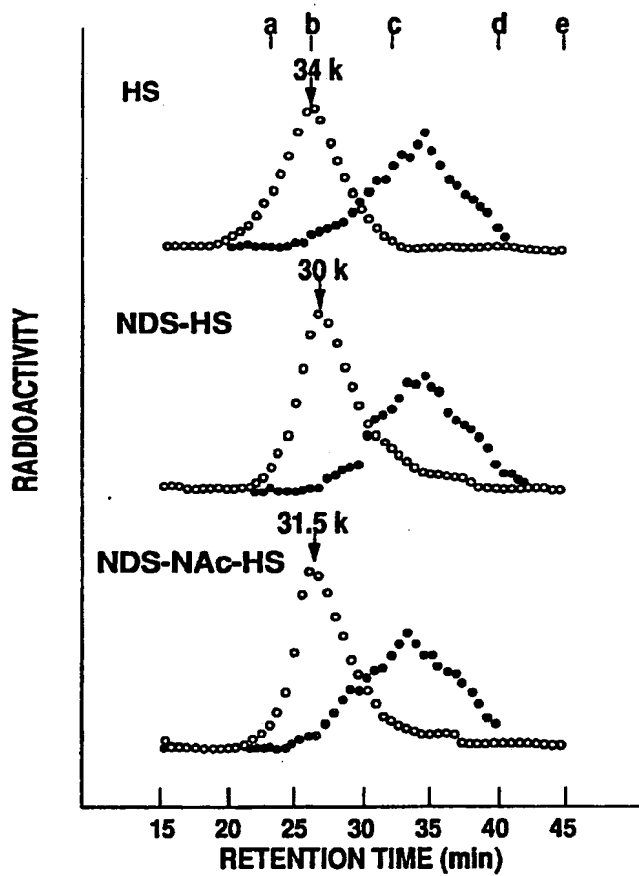


Fig. 2

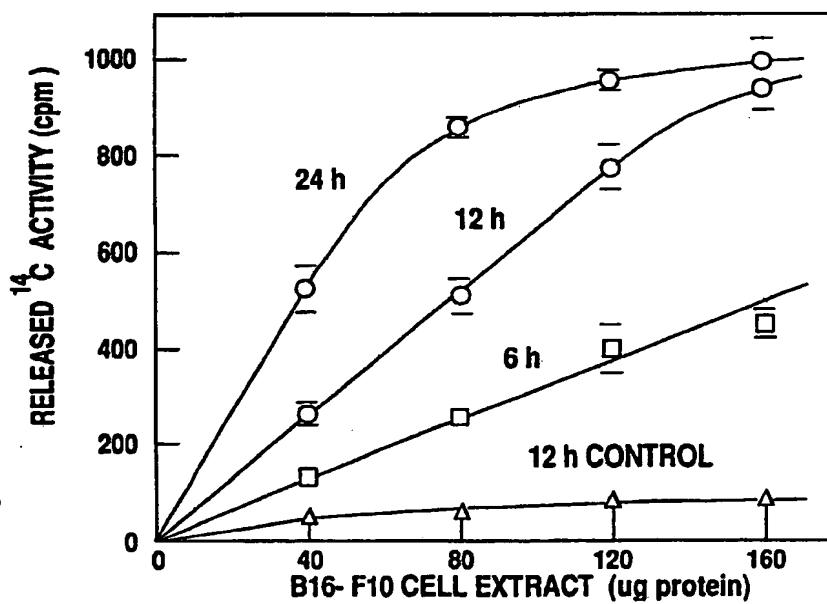


Fig.3

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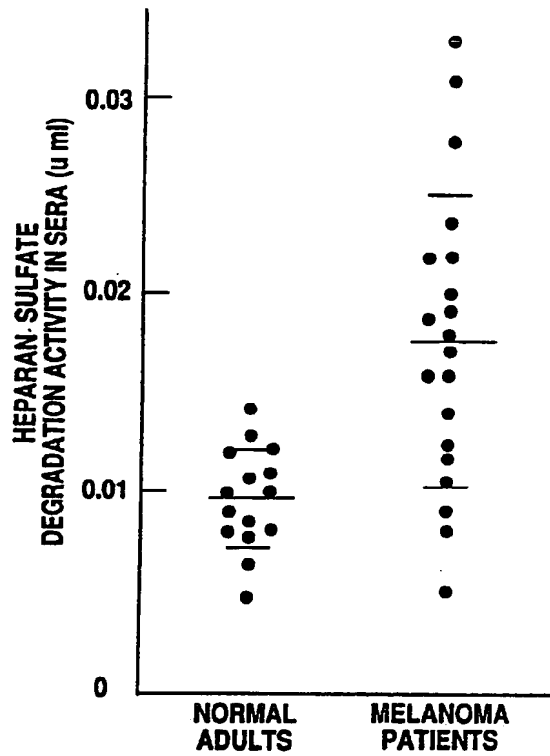


Fig. 4

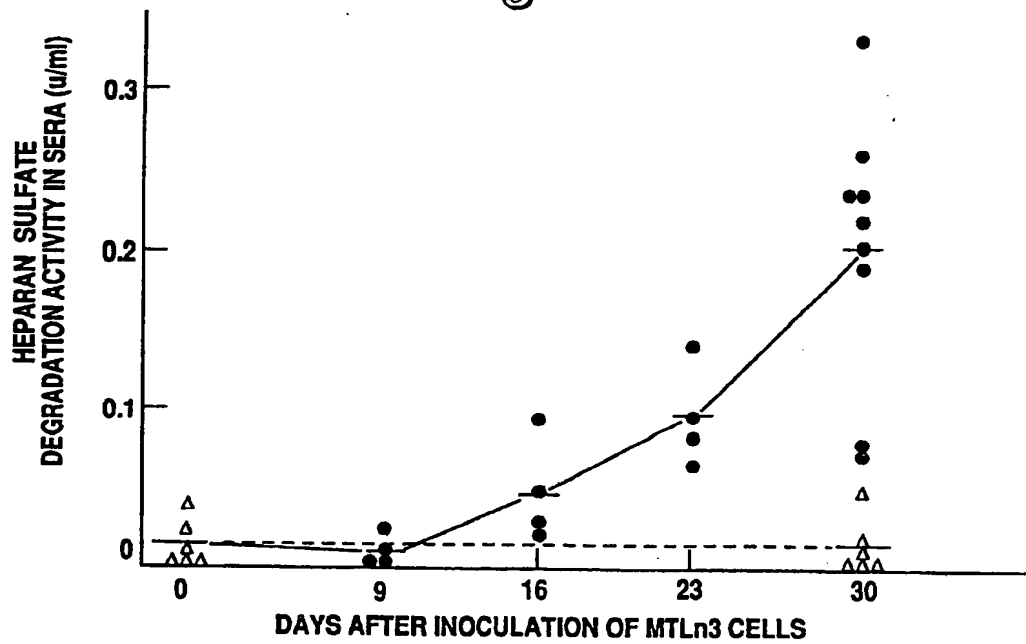


Fig. 5

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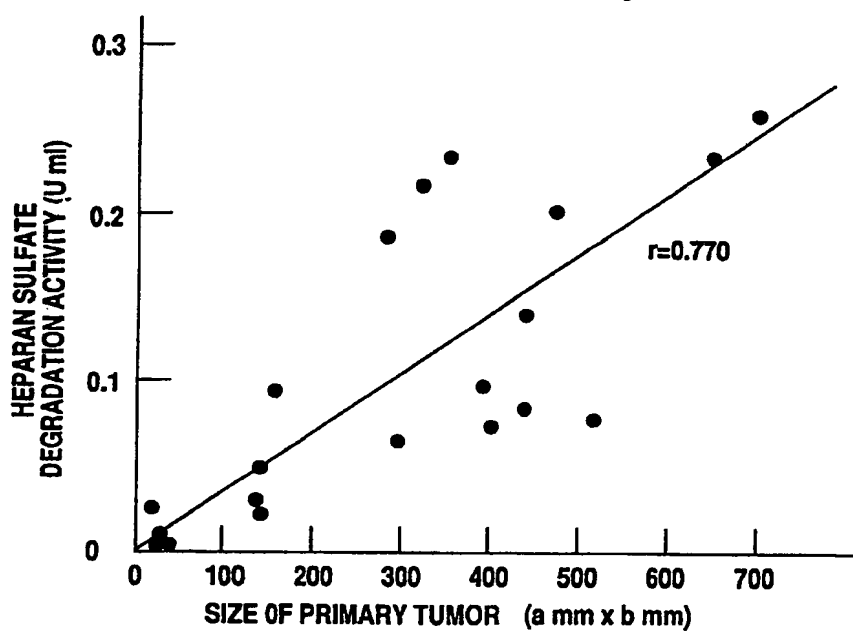


Fig.6

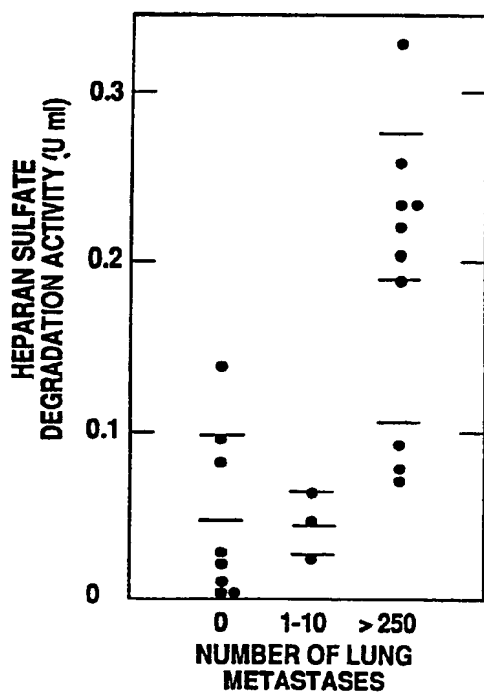


Fig.7a

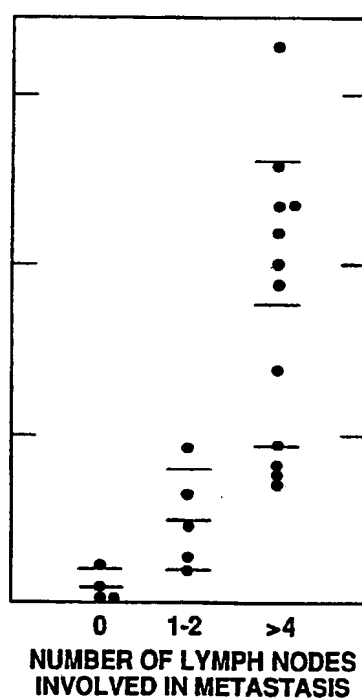


Fig.7b

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HEPARAN SULFATE PROTEOGLYCAN

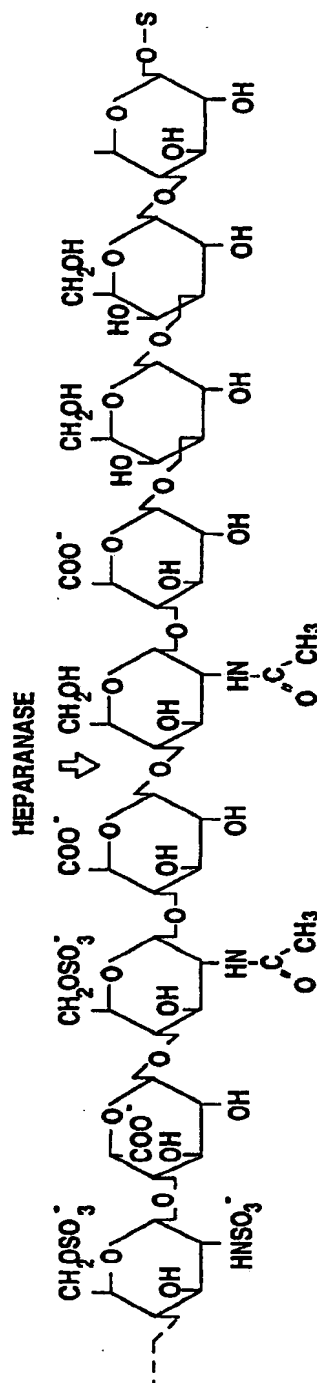
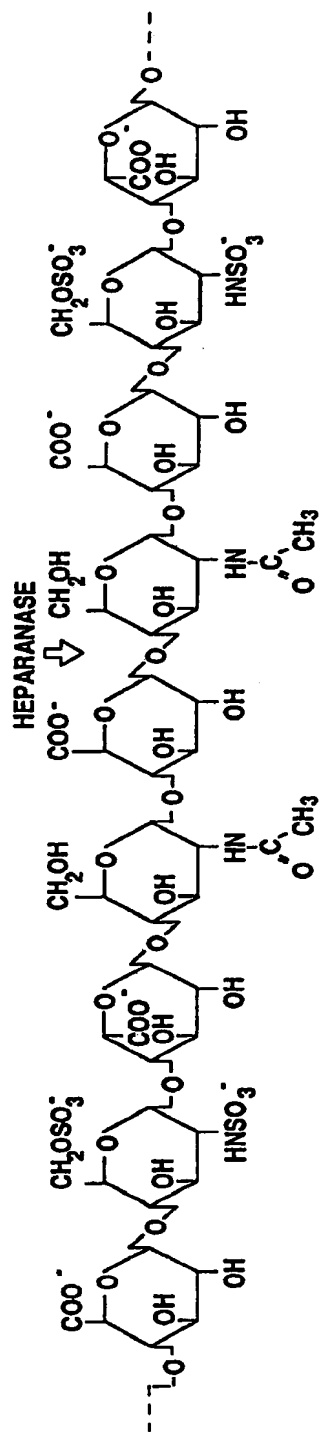


Fig. 8

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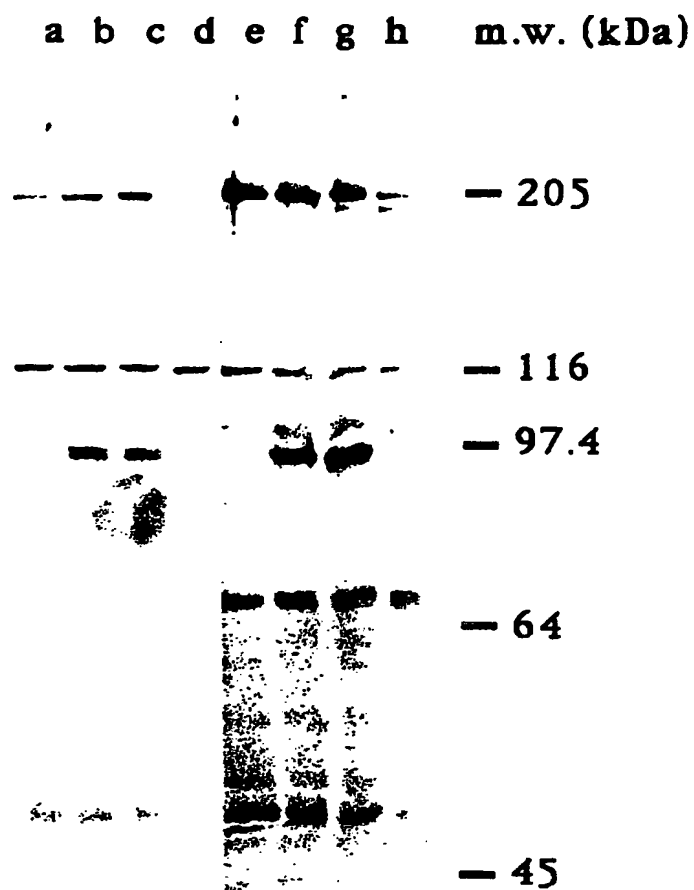


FIG. 9

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FIG. 10a



FIG. 10b



FIG. 10c



FIG. 10d



FIG. 10e

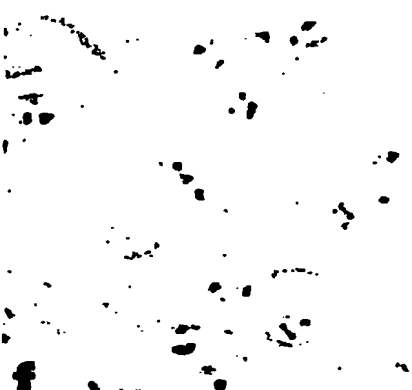


FIG. 10f

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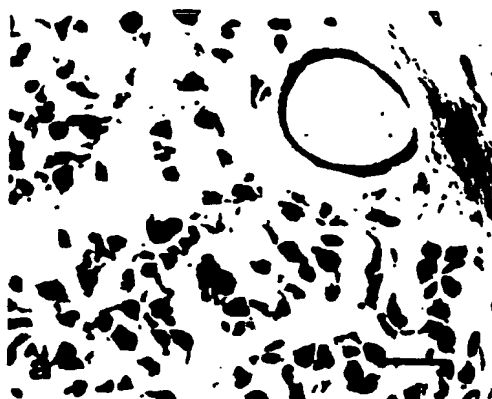


FIG. 11a

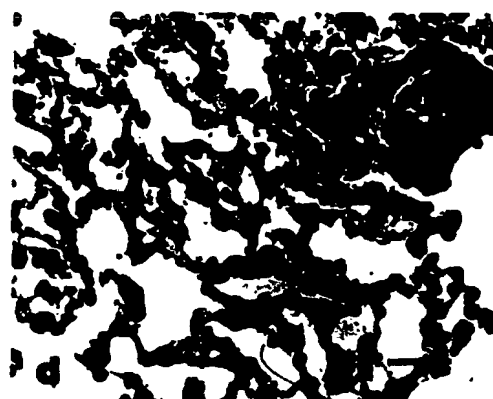


FIG. 11d

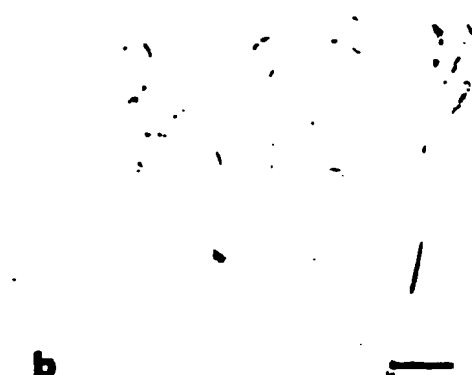


FIG. 11b



FIG. 11e

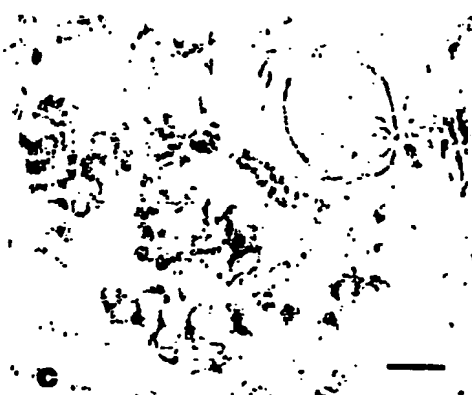


FIG. 11c



FIG. 11f

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INTERNATIONAL SEARCH REPORT

International Applicant No PCT/US 91/03832

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all) ⁶		
According to International Patent Classification (IPC) or to both National Classification and IPC Int.Cl.5 G 01 N 33/573 C 07 K 7/08 // G 01 N 33/574		
II. FIELDS SEARCHED		
Minimum Documentation Searched ⁷		
Classification System	Classification Symbols	
Int.Cl.5	G 01 N C 12 N C 07 K	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched ⁸		
III. DOCUMENTS CONSIDERED TO BE RELEVANT⁹		
Category ¹⁰	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
X	EP,A,0244932 (BOARD OF REGENTS THE UNIVERSITY OF TEXAS SYSTEM), 11 November 1987, see abstract, page 5, lines 6-12; page 8, line 24 - page 9, line 30; claims 37-39,53-59	1,2,4-21,23-26
X,P	International Journal of Cancer, vol. 45, 15 June 1990, Wiley-Liss, Inc. (US), Li Jin et al.: "Immunochemical localization of heparanase in mouse and human melanomas", pages 1088-1095, see the whole article	1-9,27-32
X,P	WO,A,9102977 (HADASSAH MEDICAL ORGANIZATION) 7 March 1991, see the abstract; claims 48-50	1,2,4-9
-/-		
<p>¹⁰ Special categories of cited documents:</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</p> <p>"Z" document member of the same patent family, priority date claimed</p>		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report	
09-10-1991	24 OCT 1991	
International Searching Authority	Signature of Authorizing Officer	
EUROPEAN PATENT OFFICE	MISS T. FAZELAAR	

Form PCT/ISA/210 (second sheet) (January 1983)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
A	Journal of Cellular Biochemistry, vol. 36, 1988, Tumor Progression and Metastasis, Alan R. Liss, Inc. M. Nakajima et al.: "Heparanases and tumor metastasis", pages 49-59, see the whole article (cited in the application) -----	1,5,6,8

Form PCT/ISA/210 (extra sheet) (January 1983)

**ANNEX TO THE INTERNATIONAL SEARCH REPORT
ON INTERNATIONAL PATENT APPLICATION NO.**

US 9103832
SA 49811

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